METABOLISM OF PHYTOALEXINS AND ANALOGS, AND INHIBITORS OF BRASSININ DETOXIFICATION IN *LEPTOSPHAERIA MACULANS*

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ABSTRACT

Blackleg disease, caused by the fungus *Leptosphaeria maculans*/*Phoma lingam*, is one of the most economically important diseases of canola and rapeseed. Detoxification of canola chemical defenses (phytoalexins and others) is an important mechanism used by the blackleg fungus to overcome the plant's natural defenses. Phytoalexins are anti-microbial defense metabolites produced *de novo* by plants in response to pathogen attack and other forms of stress. *L. maculans* is successful in detoxifying several cruciferous phytoalexins to different products. For example, brassinin, a key phytoalexin from crucifers, is transformed to indole-3-carboxaldehyde. This thesis includes investigation of phytoalexin metabolism by *L. maculans* and related work: (i) transformation pathways of cruciferous phytoalexins and analogs; (ii) design and synthesis of potential inhibitors of brassinin detoxification.

In continuation of previous work, homologues, analogs and structural relatives of brassinin were analysed for metabolism by *L. maculans*. Products of metabolism of these compounds were identified and the overall metabolic pathways were established. It was concluded that structural relatives of brassinin metabolized differently from brassinin. Antifungal bioassays of the products suggested that all these transformations were detoxification reactions. Among the phytoalexins, rapalexin A was not metabolized whereas, erucalexin was metabolized. Results of these metabolism studies using *L. maculans* along with the syntheses and antifungal activities of the metabolites will be presented.

In the second part of this thesis, inhibition of the detoxification of brassinin by *L. maculans* using quinolines and isoquinolines was investigated. These compounds resulted from replacement of indolyl containing structures with quinoline and isoquinoline moieties, and various substitutions such as phenyl, thiazolyl, bromo, chloro, hydroxyl and methoxy groups. All these compounds were tested for their effect

on brassinin detoxification and antifungal activity. Overall, a significant effect on the rate of brassinin detoxification in cultures of *L. maculans* was detected in the presence of compounds 6-bromo-2-phenylquinoline, 2-phenylquinoline, 3-phenylquinoline and 1-thiazolylisoquinoline. 6-Bromo-2-phenylquinoline was the most effective compound in slowing down the metabolism of brassinin and also was a weak inhibitor of the growth of *L. maculans* (virulent on canola). Results of the syntheses and evaluation of the compounds are discussed.

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DEDICATION

To

My Mother (1960-2002)
and Father

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Ac	Acetyl
Ac_2O	Acetic anhydride
AcOH	Acetic acid
A. brassicicola	*Alternaria brassicae*
B.	*Brassica*
B. cinerea	*Botrytis cinerea*
BOLm	Brassinin oxidase from *L. m*
BHAb	Brassinin hydrolase from *A. b*
BHLmL2	Brassinin hydrolase from *L. m* (Laird-2)
Br	Broad
^{13}C NMR	Carbon-13 nuclear magnetic resonance
C14 DM	C14 demethylase
C. albicans	*Candida albicans*
calcd.	Calculated
C. lunata	*Curvularia lunata*
Ctr	Control
Cul	Culture
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EI	Electron impact
ESI	Electrospray ionization
Et	Ethyl
EtOAc	Ethyl acetate
EtOH	Ethanol
FCC	Flash column chromatography
FTIR	Fourier transformed infrared
^{1}H NMR	Proton nuclear magnetic resonance
h	hour
Hex	Hexane
HPLC	High performance liquid chromatography
HR	High resolution

Hz	Hertz
J	Coupling constant
3 KR	Ergosterol- 3-ketoreductase
L. biglobosa	*Leptosphaeria biglobosa*
L. maculans	*Leptosphaeria maculans*
M. grisea	*Magnaporthe grisea*
m/*z*	Mass/charge ratio
Me	Methyl
MeI	Methyl iodide
MeOH	Methanol
MHz	Megahertz
MM	Minimal medium
Min	Minute(s)
NADH	Nicotinamide adenine dinucleotide (reduced)
NOE	Nuclear Overhauser effect
PDA	Potato dextrose agar
PTLC	Preparative thin layer chromatography
Pyr	Pyridine
R. solani	*Rhizoctonia solani*
RP	Reverse phase
Rt	Room temperature
SD	Scytalone dehydratase
SE	Squalene epoxidase
SsBGT1	Recombinant brassinin glucosyltransferase of *S. s*
S. cerevisiae	*Saccharomyces cerevisiae*
t_R	Retention time
THF	Tetrahydrofuran
1,3,8-THNR	1,3,8-trihydroxy naphthalene reductase
1,3,6,8-THNR	1,3,6,8-tetrahydroxy naphthalene reductase
TLC	Thin layer chromatography
UV	Ultraviolet
V	Volume

Chapter 1: Introduction

1.1 General objectives

Phytoalexins are secondary metabolites synthesized de novo by plants in response to external stress such as pathogen invasion. So far, about 44 cruciferous phytoalexins have been isolated from Brassicaceae. Pedras and co-workers investigated the detoxification of several cruciferous phytoalexins by their pathogens and determined the pathways of phytoalexin metabolism (Pedras, Yaya et al., 2011c). My research work explores the detoxification of cruciferous phytoalexins by the phytopathogenic fungus of canola *Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr) Desm. and the effects of quinoline and isoquinoline derivatives on the brassinin detoxification. The specific objectives are to:

- Determine the products of transformation of the cruciferous phytoalexins rapalexin A (**22**), erucalexin (**29**) and brussalexin (**36**) by L*eptosphaeria maculans* (isolates virulent on canola);
- Determine the products of transformation of synthetic compounds related to brassinin (**96**, **111**, **153**, **320**, **325**, **327**, **331** and **333**) by *L. maculans* (isolates virulent on canola);
- Determine antifungal activity of the phytoalexins **17**, **22**, **29**, **36** and compounds **96**, **111**, **153**, **320**, **325**, **327**, **331** and **333** against *L. maculans* (isolates virulent on canola);
- Design, synthesize and determine the antifungal activity of potential inhibitors (**350**-**366**) of brassinin detoxification based on quinoline and isoquinoline moieties;
- Determine the effect of compounds **350**-**366** on the rate of brassinin detoxification by *L. maculans* (isolates virulent on canola).

1.2 Cruciferous plants and fungal pathogens

Blackleg of cruciferous oilseeds and vegetables is an economically important disease and is a serious concern in view of the yield losses that are associated with the disease (Gugel and Petrie, 1992). Blackleg disease is caused by *L. maculans* and *L. biglobosa*. *L. biglobosa* is less virulent on canola than *L. maculans*, but could be more damaging to *Brassica juncea* (Rimmer, Shattuck et al., 2007).

Canola is defined by the Canola Council of Canada (http://www.canola-council.org) as seeds of *Brassica* genus (*B. napus*, *B. rapa* or *B. juncea*) whose oils contain < 2% erucic acid and seed meal contains < 30 µmol of aliphatic glucosinolates (any one or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate and 2-hydroxy-4-pentenyl glucosinolate) per gram of the air-dry, oil-free solid (http://www.canola-council.org/ind_definition.aspx). Canola is the second largest grown crop in Saskatchewan. In 2007, four million tonnes of canola was produced in Saskatchewan, which amounted to 45% of Canada's total production (Source: http://www.agriculture.gov.sk.ca/Saskatchewan_Picture). Blackleg disease is a major concern in canola growing areas all over the world. However, disease management practices such as the use of disease resistant cultivars, crop rotation, management of infested stubble, use of disease-free seeds has proved to be effective (Gugel and Petrie, 1992; Rimmer, Shattuck et al., 2007).

Seeds are the major source of spread of blackleg as *L. maculans* can survive in seeds for long time. Hence, disease-free seeds obtained by fungicidal treatment were found effective for controlling blackleg disease (Gugel and Petrie, 1992). In Canada, carbathin, thiram and iprodione are registered fungicides used in seed treatments (West, Kharbanda et al., 2001). Besides, azoxystrobin, fludloxanil, metalaxyl and other fungicidal mixtures are recommended for the purpose (Source: www.ag.ndsu.edu). Despite the measures taken, statistics from the Canadian plant disease survey of 2010 showed that blackleg infections were found in 55% of the tested canola fields and that the mean incidence of the disease has not decreased in Saskatchewan over last 10 years

(Dokken-Bouchard, Anderson et al., 2011). These statistics suggest that better practices are required for the control of blackleg disease.

1.3 Secondary metabolites of cruciferous plants and blackleg fungi in attack and defense

Interactions among plants and pathogens are complex and involve attack and counterattack mechanisms (Hahlbrock, Bednarek et al., 2011). Secondary metabolites of fungi and plants play a major role in these interactions (Berger, Sinha et al., 2007). In general, fungal invasion of the plant involves production of toxins and other metabolites by the pathogen, which damages the plant cells irreversibly. During the infection process, some of the plant receptor sites can recognize fungal metabolites (elicitors) that induce defense responses such as production of antifungal compounds (Pedras, 2011; Berger, Sinha et al., 2007). Besides, both plants and pathogens produce enzymes to detoxify the toxic chemicals produced by the opponent. Thus, there exists a continuous arms race between plants and fungal pathogens (Pedras, 2011). It is important to understand the chemistry and biochemistry involved in these interactions to find better ways to protect plants.

1.3.1 Fungal metabolites

Crucifer pathogens produce structurally diverse metabolites. Some of them are known to be host-selective phytotoxins, which cause disease symptoms only on the plants that host the fungal species (Pedras and Yu, 2009). The actual role of these phytotoxins/ metabolites in fungal defense is not well understood. However, some of the metabolites are characteristic of a particular fungal species (Pedras, Chumala et al., 2007a). Metabolites produced by various fungal pathogens of crucifer crops were reported for *Alternaria brassicicola* (Pedras, Chumala et al., 2009a), *Rhizoctinia solani* (Pedras, Yu et al., 2005a) and *Sclerotinia sclerotiorum* (Pedras and Ahiahonu, 2004). Metabolite production by *L. maculans* was extensively studied and has been reviewed (Pedras and Yu, 2009; Pedras, 2001). The blackleg disease is caused by two species *L. maculans*

and *L. biglobosa*. *L. maculans* has several sub groups known to produce quite different metabolites. Isolates of *L. maculans* virulent on canola (Pedras and Yu, 2009) and mustard (Pedras, Chumala et al., 2004b; Pedras and Yu, 2009) produce several metabolites and phytotoxins.

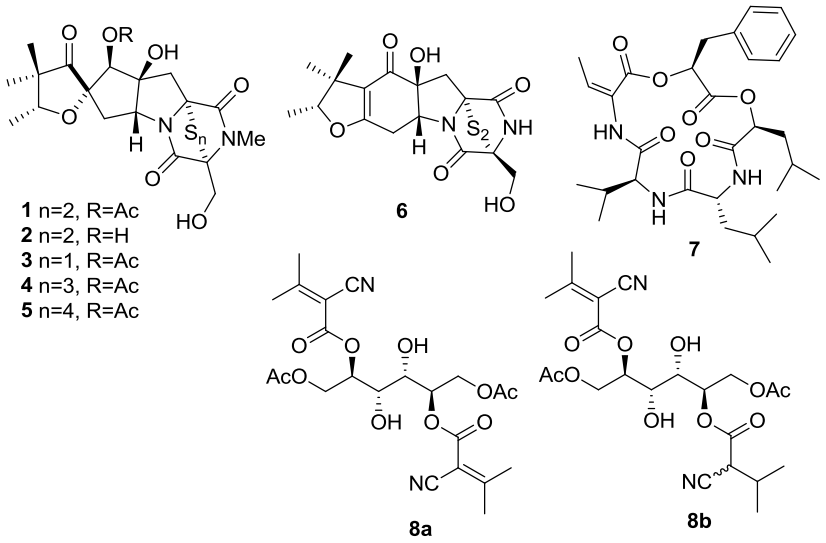


Figure 1.1 Selected structures of phytotoxins: sirodesmins **1-5**, phomalirazine (**6**), phomalide (**7**), maculansins **8a** and **8b** produced by *Leptosphaeria maculans* (virulent on canola) (Pedras and Yu, 2009; Pedras, Chumala et al., 2007a).

Sirodesmin PL (**1**) and deacetylsirodesmin PL (**2**) were the first phytotoxins isolated from *L. maculans* (virulent on canola) (Ferezou, Riche et al., 1977). Since then, other phytotoxins such as sirodesmin H (**3**), sirodesmins J (**4**) and K (**5**) belonging to this family have been isolated from cultures of *L. maculans* (virulent on canola) grown in minimal medium (MM) (Pedras, Seguinswartz et al., 1990). All these sirodesmins, which are polythiodioxopiperazines, cause yellow necrotic lesions on leaves of different plants, including crucifers (Pedras, Chumala et al., 2007a). Phomalirazine (**6**) was also produced by isolates of *L. maculans* virulent on canola. Compound **6** caused nectrotic lesions on both susceptible and resistant plants (Pedras, Abrams et al., 1989). Phomalirazine (**6**) was a proposed intermediate in the biosynthesis of sirodesmin PL (**1**) (Pedras, Abrams et al., 1989). Phomalide (**7**) is the first host-selective phytotoxin isolated from cultures of *L. maculans* (virulent on canola) grown in MM (Pedras,

Taylor et al., 1993). Compound **7** caused necrotic lesions on canola leaves but not on those of brown mustard (Pedras and Yu, 2009; Pedras and Biesenthal, 1998). The biosynthesis of phomalide (**7**) was inhibited in the presence of sirodesmin PL (**1**) (Pedras and Biesenthal, 1998). Maculansin A (**8a**) was identified as major metabolite from cultures of *L. maculans* (isolates virulent on canola) grown in PDB medium (Pedras and Yu, 2008). Compound **8a** caused lesions on both susceptible and resistant plants (Pedras and Yu, 2008; Pedras, 2011). The phytotoxins produced by *L. maculans* (isolates virulent on canola) are shown in figure **1.1**.

Depsilardin (**9**) is a host-selective toxin produced by isolates of *L. maculans* (virulent on mustard). Compound **9** caused nectrotic lesions when sprayed on leaves of brown mustard but not canola (Pedras, Chumala et al., 2004b). The total synthesis of compound **9** was recently accomplished (Ward and Pardeshi, 2010). Polanrazines B (**10**) and C (**11**) were identified as phytotoxic among the group of structurally similar compounds produced by Polish type isolates of *L. maculans* (Pedras and Biesenthal, 2001). Phomalairdenol A (**12**) and phomalairdenones A (**13**) and D (**14**) were also isolated from isolates of *L. maculans* (virulent on mustard) (Figure **1.2**) (Pedras, Erosa-Lopez et al., 1999a).

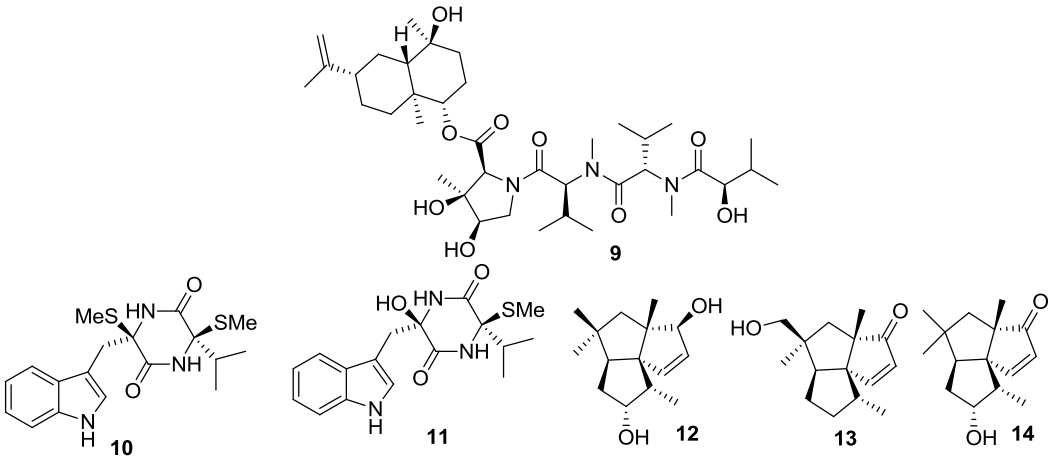


Figure 1.2 Selected structures of phytotoxins **9**-**14** produced by *Leptosphaeria maculans* (virulent on mustard) (Pedras and Yu, 2009) .

There is another group of fungal metabolites known as elicitors. Elicitors bind to receptor sites in plant tissues and induce production of phytoalexins (Hahn, 1996).

Phytoalexins are antimicrobial plant metabolites produced de novo upon exposure to biotic and abiotic stresses (Bailey and Mansfield, 1982). A mixture of cerebrosides C (**15**) and D (**16**) are the only known specific elicitors isolated from *L. maculans* (virulent on canola) (Figure **1.3**). In addition, sirodesmin PL (**1**) and deacetylsirodesmin PL (**2**) were identified as non-specific elicitors (Pedras and Yu, 2009).

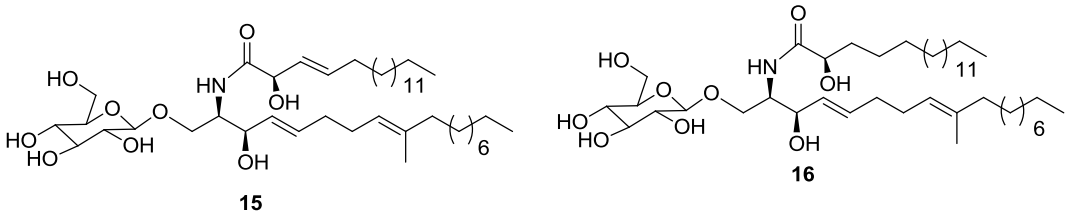


Figure 1.3 Elicitors **15** and **16** produced by *Leptosphaeria maculans* (virulent on canola) (Pedras and Yu, 2009) .

1.3.2 Cruciferous plant metabolites

Phytoalexins (inducible) (Bailey and Mansfield, 1982) and phytoanticipins (constitutive) (Vanetten, Mansfield et al., 1994) play important ecological roles in defense against pathogens (Hammond-Kosack and Jones, 1996). Phytoanticipins are constitutive plant defenses, whose concentrations increase under stress conditions (Vanetten, Mansfield et al., 1994). The developments in cruciferous phytoalexin research have been reviewed recently (Pedras, Yaya et al., 2011c; Pedras and Yaya, 2010; Pedras, 2008; Pedras, Zheng et al., 2007e; Pedras and Ahiahonu, 2005; Pedras, Jha et al., 2003b; Pedras, Okanga et al., 2000).

Phytoalexins

Recently, Pedras et al., published a comprehensive review dealing with cruciferous phytoalexins (Pedras, Yaya et al., 2011c). For this reason, only work of immediate interest to this thesis will be reviewed here. Phytoalexins are generally found at the site of infection at inhibitory concentrations. Localization studies carried out on several species infected with the pathogens illustrated that phytoalexins have specific roles in defense (Hammerschmidt, 1999).

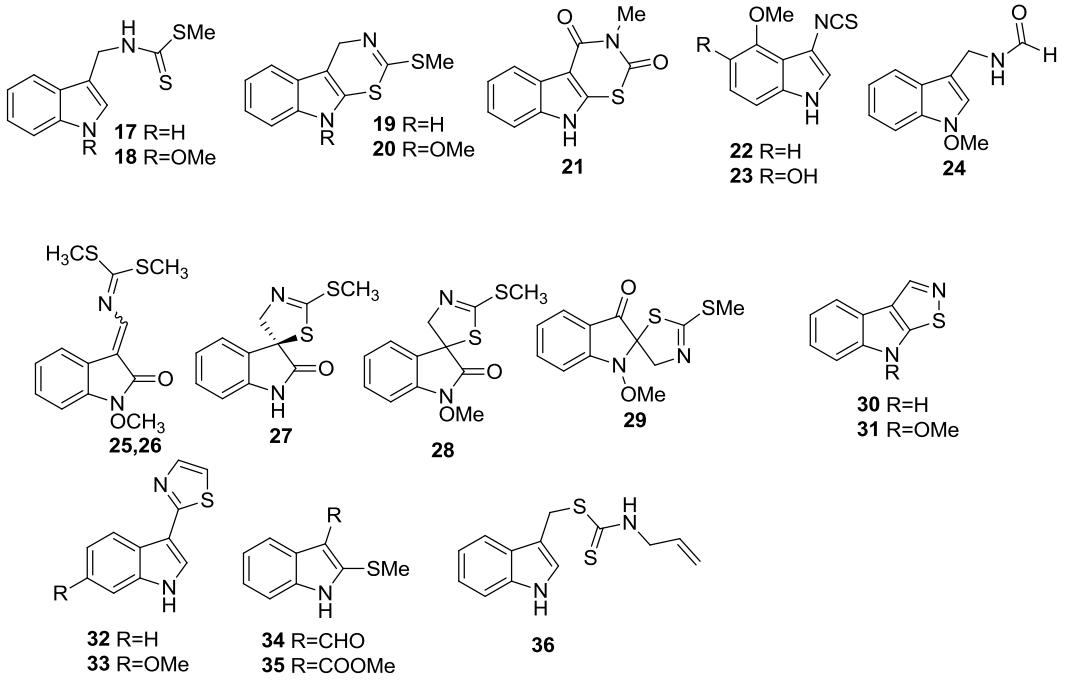
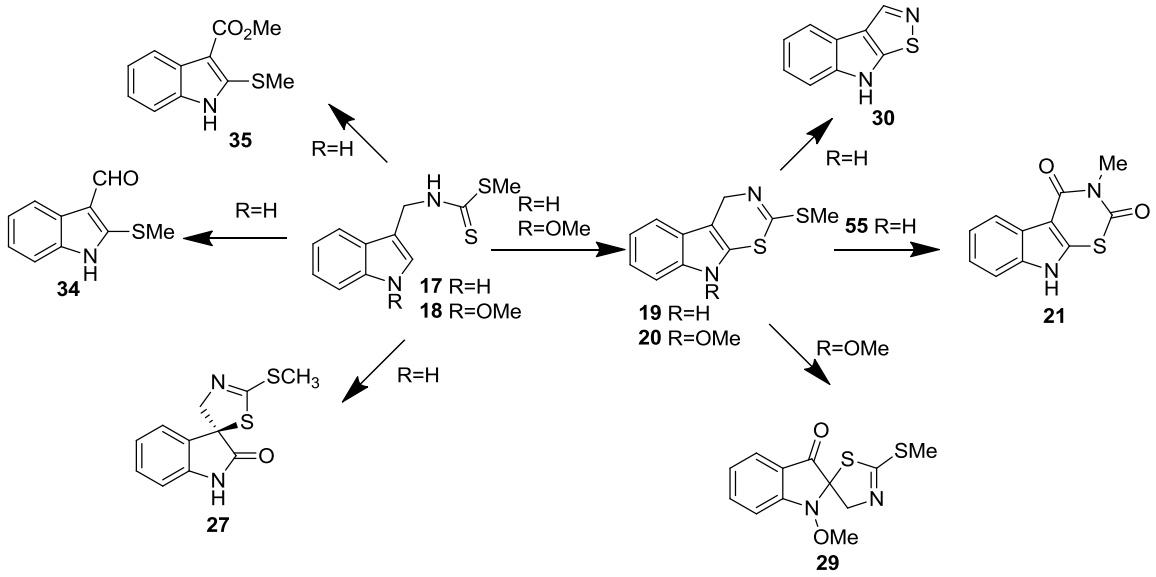


Figure 1.4 Structures of selected cruciferous phytoalexins: brassinin (**17**), 1-methoxybrassinin (**18**), cyclobrassinin (**19**), 1-methoxycyclobrassinin (**20**), rutalexin (**21**), rapalexin A (**22**), rapalexin B (**23**), caulilexin B (**24**), wasalexins A (**25**) and B (**26**), spirobrassinin (**27**), 1-methoxyspirobrassinin (**28**), erucalexin (**29**), brassilexin (**30**), sinalexin (**31**), camalexin (**32**), 6-methoxycamalexin (**33**), brassicanal A (**34**), brassicanate A (**35**) and brussalexin (**36**) (Pedras, Yaya et al., 2011c).

The phytoalexins brassinin (**17**) and 1-methoxybrassinin (**18**) were isolated from Chinese cabbage by Takasugi and coworkers in 1986 and these compounds were the first phytoalexins reported from Brassicaceae (Takasugi, Katsui et al., 1986). Since then, 44 cruciferous phytoalexins have been isolated and their structures have been elucidated (Pedras, Yaya et al., 2011c). Crucifers belong to the only plant family known to date to produce sulfur-containing phytoalexins. Phytoalexins such as brassinins **17** and **18** contain a dithiocarbamate group. The dithiocarbamate group was present in several fungicides used in 20th century (Atkinson, 1970). Wasalexins **25**, **26** and camalexins **32** and **33** were isolated from wild crucifers (Pedras, Yaya et al., 2011c). Selected structures of cruciferous phytoalexins **17**-**36** are shown in figure **1.4**. Cruciferous phytoalexins are also known to exhibit broad spectrum of antifungal activities against several pathogens (Pedras, Yaya et al., 2011c). The phytoalexin

brassinin (**17**) is a strong inhibitor of the growth of *L. maculans*, *S. sclerotiorum* and *B. cinerea*. Brassinins **17** and **18** are considered crucial metabolites among the cruciferous phytoalexins, because they are precursors of several other phytoalexins. Scheme **1.1** summarizes the biosynthetic relationships among the various cruciferous phytoalexins (Pedras, Yaya et al., 2011c).



Scheme 1.1 Biosynthetic relationship among brassinins **17** and **18**, and other phytoalexins cyclobrassinin (**19**), 1-methoxycyclobrassinin (**20**), rutalexin (**21**), spirobrassinin (**27**), erucalexin (**29**), brassilexin (**30**), brassicanal A (**34**), brassicanate A (**35**) (Pedras, Yaya et al., 2011c).

Phytoanticipins

Phytoanticipins are constitutive plant metabolites whose concentrations increase after the stress conditions are applied (Vanetten, Mansfield et al., 1994). The distinction between phytoalexins and phytoanticipins is sometimes ambiguous, as the same compound can be a phytoanticipin in one species and be inducible (phytoalexin) in others. For example, arvelexin (**39**) was isolated from the wild crucifer *Thlapsi arvense* as a phytoalexin (Pedras, Chumala et al., 2003a), while compound **39** was isolated as phytoanticipin from canola treated with *Plasmodiophora brassicae* (Pedras, Zheng et al., 2008b). Methyl 1-methoxyindole-3-carboxylate (**41**) was first isolated as a

phytoalexin from wasabi (Pedras, Sorensen et al., 1999b), whereas the same compound was isolated as a phytoanticipin in *Arabidopsis thaliana* (Pedras and Adio, 2008). Compounds **38**-**40** and **42** are phytoanticipins detected in *A. thaliana* (Pedras and Adio, 2008). Phytoanticipins **43** and **44** were isolated from oilseed canola infected with *P. brassicae* (Pedras, Zheng et al., 2008b). Besides, ascorbigen (**45**), neoascorbigen (**46**), dihydroascorbigens **47**, **48** and indole glucosinolates **49**-**51** were the other phytoanticipins isolated (Figure **1.5**) (Pedras and Adio, 2008).

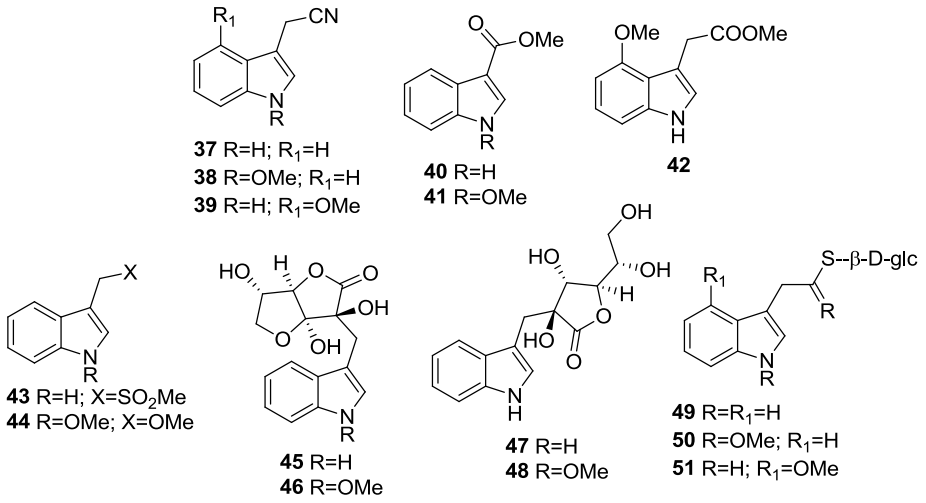


Figure 1.5 Structures of phytoanticipins: indole-3-acetonitriles **37**-**39**, compounds **40**-**44**, ascorbigens **45**-**48** and glucobrassicins **49**-**51**, produced by cultivated crucifers (Pedras, Zheng et al., 2008b) and wild crucifers (Pedras and Adio, 2008).

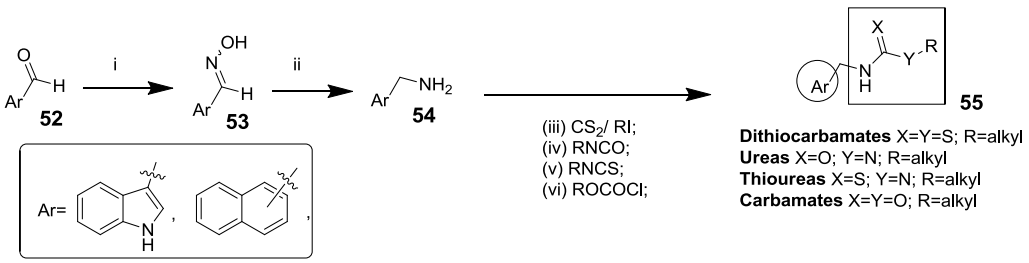
Several phytoanticipins of Brassicaceae are indole glucosinolates **49**-**51** and their degradation products **37**-**39**. Glucosinolates are derived from aminoacids and are produced by several plant families. Grouping of 120 cruciferous glucosinolates into 10 (A-J) groups by Fahey and co-workers (Fahey, Zalcmann et al., 2002) was recently revised by Clarke. Clarke compiled a list of 200 glucosinolates into 13 (A-M) groups (Clarke, 2010).

1.4 Biotransformation of cruciferous phytoalexins and related structures by phytopathogenic fungi

Fungal pathogens produce detoxifying enzymes, which metabolize plant defense compounds such as phytoalexins to less toxic products (Pedras, Yaya et al., 2011c; Pedras and Yaya, 2010; Pedras, 2008; Pedras and Ahiahonu, 2005). The metabolism of crucifer phytoalexins by several pathogens such as *L. maculans*, *L. biglobosa*, *A. brassicicola*, *S. sclerotiorum* and *R. solani* were reviewed (Pedras, Yaya et al., 2011c; Pedras, 2008; Pedras and Ahiahonu, 2005; Pedras, Okanga et al., 2000). However, the metabolism of the compounds related to phytoalexins has not been covered in these reviews. Thus, the synthesis and metabolism of compounds related to brassinin (**17**), brassilexin (**30**) and camalexin (**32**) by pathogens such as *L. maculans* (virulent on canola), *L. biglobosa*, *A. brassicicola*, *S. sclerotiorum* and *R. solani* will be reviewed in this section.

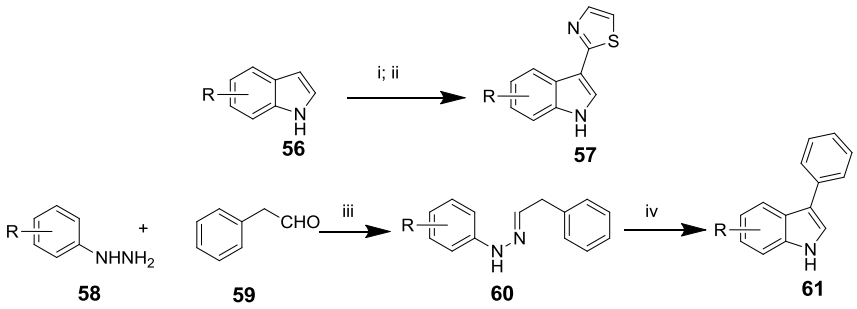
1.4.1 Syntheses of phytoalexin-related structures

In this section, general routes used in the synthesis of analogs and related structures of brassinin (**17**), brassilexin (**30**) and camalexin (**32**) will be discussed. Compounds related to brassinin (**17**) with changes in the aryl moiety and functional group were prepared to test as inhibitors of brassinin detoxification (Pedras and Jha 2006). Similar to brassinin, their syntheses were initiated with oximation of aldehydes of general structure **52** in presence of $NH_2OH.HCl$ to yield oximes of general structure **53**, which were reduced to the corresponding amines of general structure **54**. Treatment of these amines **54** with different reagents (iii-vi) as shown in scheme **1.2** afforded the compounds of general structure **55**, which included dithiocarbamates, ureas, thioureas and carbamates (Pedras and Jha, 2006).



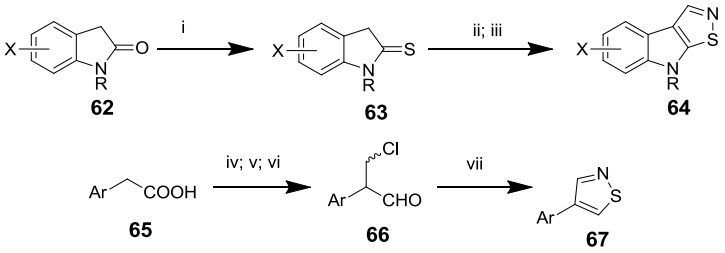
Scheme 1.2 Synthesis of brassinin analogs of general structure **55**. Reagents and conditions: (i) $NH_2OH.HCl$, Na_2CO_3, EtOH; (ii) $NiCl_2.6H_2O$, $NaBH_4$, MeOH; (iii) CS_2, Pyr, Et_3N, alkyliodide; (iv) Et_3N, alkyl/aryl isocyanate; (v) Et_3N, alkyl/aryl isothiocyanate; (vi) Et_3N, alkylchloroformate (Pedras and Jha, 2006).

A general procedure by Ayer and co-workers (Ayer, Craw et al., 1992) was used to synthesize substituted camalexins of general structure **57**. Solutions of 2-bromothiazole in benzene were added to indoles of general structure **56** and MeMgI, and refluxed to afford camalexins **57** (Pedras and Ahiahonu, 2002; Pedras and Liu, 2004). Similarly, Fischer indole synthesis was used to synthesize the substituted 3-phenylindoles of general structure **61** (Scheme **1.3**) (Robinson, 1963). Substituted phenyl hydrazines of general structure **58** and phenyl acetaldehydes of general structure **59** were heated to give the hydrazine intermediates of general structure **60**, which were heated in EtOH in presence of $ZnCl_2$ to afford the substituted 3-phenylindoles **61** (Pedras and Hossain, 2007).



Scheme 1.3 Synthesis of camalexin analogs of general structure **57** and **61**. Reagents and conditions: (i) MeMgI, Et_2O; (ii) 2-bromothiazole, benzene (Ayer and Craw et al., 1992; Pedras and Ahiahonu, 2002; Pedras and Liu, 2004); (iii) heat; (iv) $ZnCl_2$, EtOH, heat (Pedras and Hossain, 2007).

Some of the analogs of brassilexin (**30**) reported in the literature have various substituents on the indole ring. Synthesis of brassilexins of general structure **64** was carried out using an optimized procedure (Pedras and Jha, 2005). Indoline-2-thiones of general structure **63** obtained from thionation of various substituted oxindoles of general structure **62** were subjected to Vilsmeier formylation followed by aqueous ammonia work-up to afford the substituted brassilexins **64**. Other brassilexin related compounds of general structure **67** with isothiazolyl moieties in their structure were synthesized according to the general procedure (Pedras and Suchy, 2006) (Scheme **1.4**). Aryl acetic acids of general structure **65** were converted to substituted aryl acrylaldehydes of general structure **66**, which in presence of NH_4SCN were cyclized to isothiazoles **67**.



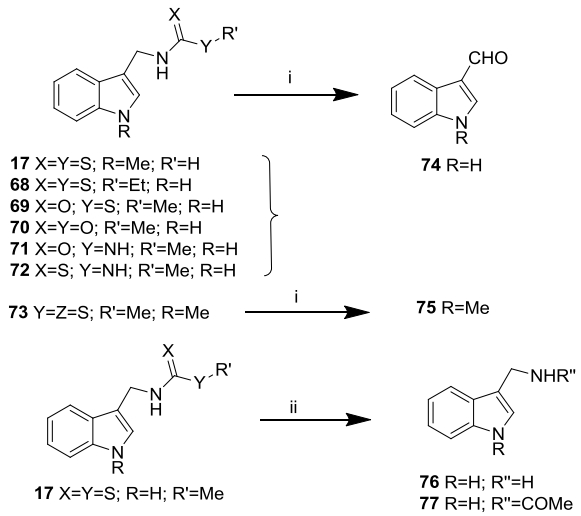
Scheme 1.4 Synthesis of brassilexin analogs of general structure **64** and **67**. Reagents and conditions: (i) P_4S_{10}, $NaHCO_3$, THF; (ii) $POCl_3$, DMF, NH_4OH; (iii) I_2, Pyr, (Pedras and Jha, 2005); (iv) $POCl_3$, DMF; (v) NaOH, $EtOH/H_2O$; (vi) $SOCl_2$, DCM; (vii) NH_4SCN, DMF, (Pedras and Suchy, 2006).

1.4.2 Biotransformations in fungal cultures

Detoxification is a general strategy used by organisms to convert toxic compounds to less toxic products. Detoxification processes involve chemical modifications or degradation of the compound. In general, the first step in the metabolism is the detoxification process. The range of transformations of phytoalexins by crucifer pathogens is very broad as shown in several reviews (Pedras, Yaya et al., 2011c; Pedras, 2008; Pedras and Ahiahonu, 2005; Pedras, Okanga et al., 2000). Besides

phytoalexins, several related compounds have been probed for metabolism by crucifer pathogens. In this section, first the transformations of compounds closely related to brassinin (**17**) are reviewed. Then, examples highlighting the metabolic transformations carried out by fungal pathogens of crucifers are illustrated using analogs of brassilexin (**30**) and camalexin (**32**).

Brassinin (**17**) is antifungal against *L. maculans*, *B. cinerea* and *S. sclerotiorum*, and more importantly, compound **17** is a biosynthetic precursor of other phytoalexins (Scheme **1.1**) (Pedras, Yaya et al., 2011c). Thus, brassinin (**17**) is considered as an important compound among crucifer defenses. Metabolism of brassinin (**17**) has been extensively studied in crucifer pathogens and these studies led to the isolation of four brassinin detoxifying enzymes BOLm (Pedras, Minic et al., 2008a), BHLmL2 (Pedras, Minic et al., 2009b), BHAb (Pedras, Minic et al., 2009c) and SsBGT1 (recombinant) (Sexton, Minic et al., 2009). In fact, the metabolism of compound **17** to indole-3-carboxaldehyde (**74**) by *L. maculans* was the first detoxification of phytoalexins identified in cruciferous pathogenic fungi (Pedras and Taylor, 1991). Later, several analogs of compound **17** have been probed for the metabolism by *L. maculans*. These compounds were designed by modifying the structure of brassinin (**17**): i) replacement of the indolyl ring in structure **17** with other aromatic moieties; ii) isosteric replacement of the hetero atoms in dithiocarbamate side chain (-N-C(S)-SMe) in the structure **17** to incorporate thiourea, carbamate, carbonate, ester and thiocarbamate analogs (Pedras and Jha, 2006). Based on these results, structural features required for metabolism of brassinin-related compounds by *L. maculans* were highlighted (Pedras, Jha et al., 2007c). For brassinin-like structures to be metabolized through oxidative degradation by *L. maculans*, the compounds were required to have at least a methylene bridge separating the aromatic moiety and the dithiocarbamate side chain. Compounds **68**-**73** were metabolized in a similar manner to compound **17** (Scheme **1.5**). However, brassinin (**17**) was metabolized differently by *L. maculans* (virulent on mustard) (Pedras, Gadagi et al., 2007b).

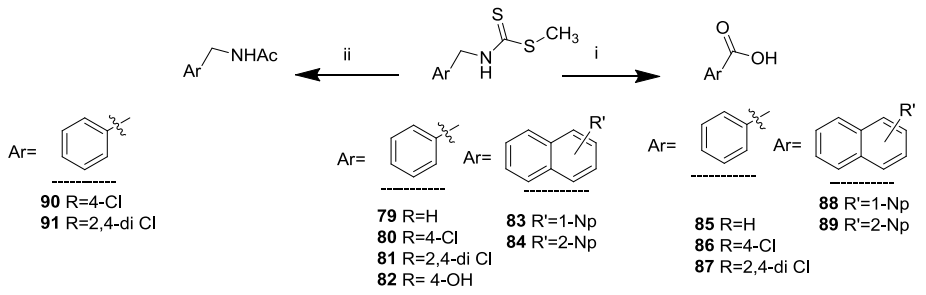


Scheme 1.5 Biotransformation of brassinin (**17**) and analogs **68**-**73** by (i) *Leptosphaeria maculans* (virulent on canola) (Pedras and Jha, 2006; Pedras, Jha et al., 2007c); (ii) *Leptosphaeria maculans* (virulent on mustard) (Pedras, Gadagi et al., 2007b; Pedras, Minic et al., 2009b).

Isolates of *L. biglobosa* and *L. maculans* (virulent on mustard) metabolized brassinin (**17**) to 3-indolylmethanamine (**76**) followed by N_b-acetyl-3-indolylmethanamine (**77**) (Pedras, Gadagi et al., 2007b). Similar to brassinin (**17)**, compounds **68**-**73** were metabolized giving the respective acetylated amines **77** or **78** (Scheme **1.5**) (Pedras, Gadagi et al., 2007b; Pedras, Minic et al., 2009c).

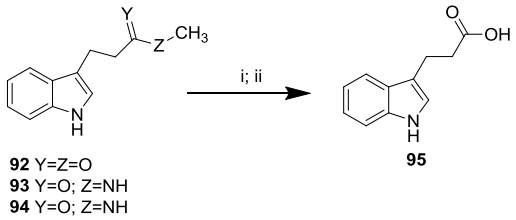
Compounds **79**-**84** were metabolized to the corresponding acids **85**-**89** in cultures of *L. maculans* (Scheme **1.6**) (Pedras and Jha, 2006; Pedras, Khan et al., 1997). The corresponding aldehydes were not detected in fungal cultures, unlike with the metabolism of compound **17** (Pedras and Jha, 2006; Pedras, Khan et al., 1997).

However, transformations of the non-indolyl dithiocarbamates such as compounds **79**, **81** and compound **17** were found to be similar in *L. maculans* (virulent on mustard). Compounds **79** and **81** were metabolized to respective products of dithiocarbamate hydrolysis followed by acetylation to yield the final products **90** and **91** (Scheme **1.6**) (Pedras, Khan et al., 1997).



Scheme 1.6 Biotransformation of dithiocarbamate analogs **79**-**84** by (i) *Leptosphaeria maculans* (virulent on canola) (Pedras and Jha, 2006; Pedras, Khan et al., 1997); (ii) *Leptosphaeria biglobosa* (Pedras, Khan et al., 1997)**.**

Both virulent and avirulent isolates of *L. maculans* metabolized compound **92** in identical manner. Compounds **93** and **94** were also hydrolyzed to indolyl-3-propanoic acid (**95**) in cultures of *L. maculans* (virulent on canola) (Scheme **1.7**).

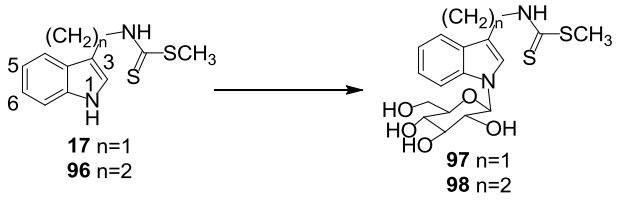


Scheme 1.7 Biotransformation of brassinin analogs **92**-**94** by (i) *Leptosphaeria maculans* (virulent on canola) (Pedras and Jha, 2006; Pedras, Jha et al., 2007c); (ii) *Leptosphaeria maculans* (virulent on mustard) (Pedras, Gadagi et al., 2007b).

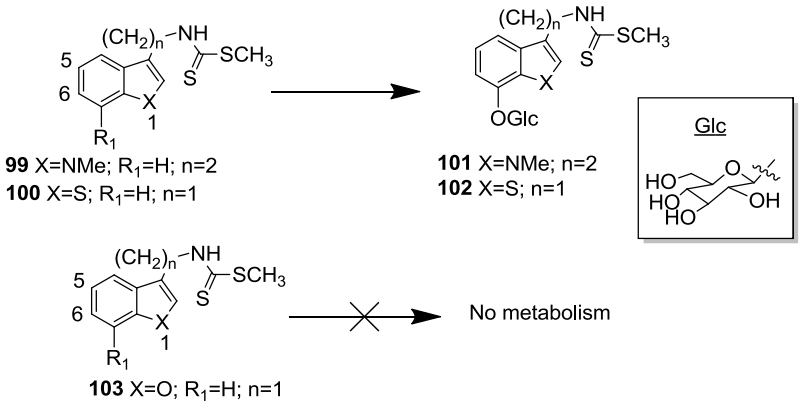
Metabolic detoxification of brassinin (**17**) in *S. sclerotiorum* was first reported in 2004 (Pedras, Ahiahonu et al., 2004a). Brassinin (**17**) and its analog **96** were metabolized similarly to products **97** and **98**, adding glycosyl moiety at N-1 in both the compounds (Scheme **1.8**) (Pedras, Ahiahonu et al., 2004a). *S. sclerotiorum* carried out the glycosylation of *S*-methyl 1-methyltryptamine dithiocarbamate (**99**) at C-7 of indole, since position C-1 of indole was protected with a $-CH_3$ group. Compound **101** was the final product of the transformation of **99** by *S. sclerotiorum* (Ahiahonu, Ph. D. Thesis, 2004). Similar transformation of compound **100** to compound **102** was observed in cultures of *S. sclerotiorum* incubated with **100**, which has the N atom replaced by S at position 1 of indole (Pedras and Hossain, 2007). When S in compound **100** at position

1 was replaced by O, the resultant compound **103** was resistant to degradation by *S. sclerotiorum* (Scheme **1.9**) (Pedras and Hossain, 2007).

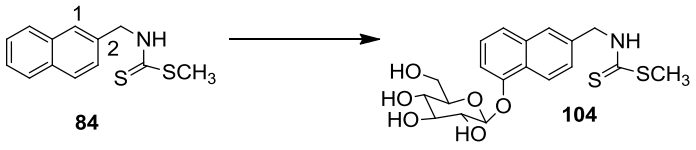
Metabolism of compound **84** in *S. sclerotiorum* yielded compound **104** as a result of glycosylation at position 5 of the naphthyl ring (Scheme **1.10**) (Pedras, Ahiahonu et al., 2004a). Examples in schemes **1.9** through **1.11** suggest that glycosylation of phytoalexins or analogs is the main transformation carried out by *S. sclerotiorum* for detoxification.



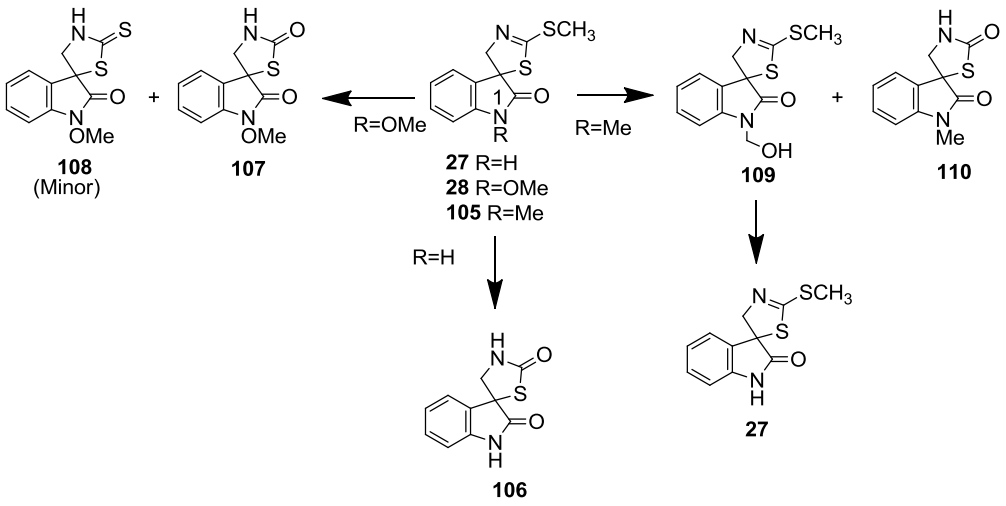
Scheme 1.8 Metabolism of brassinin (**17**) and analog **96** by *Sclerotinia sclerotiorum* (Pedras, Ahiahonu et al., 2004a).



Scheme 1.9 Metabolism of compounds **99** and **100** by *Sclerotinia sclerotiorum* (Pedras and Hossain, 2007).

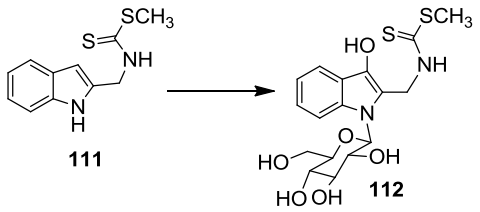


Scheme 1.10 Detoxification of methyl 2-naphthylmethyl dithiocarbamate (**84**) by *Sclerotinia sclerotiorum* (Pedras, Ahiahonu et al., 2004a).



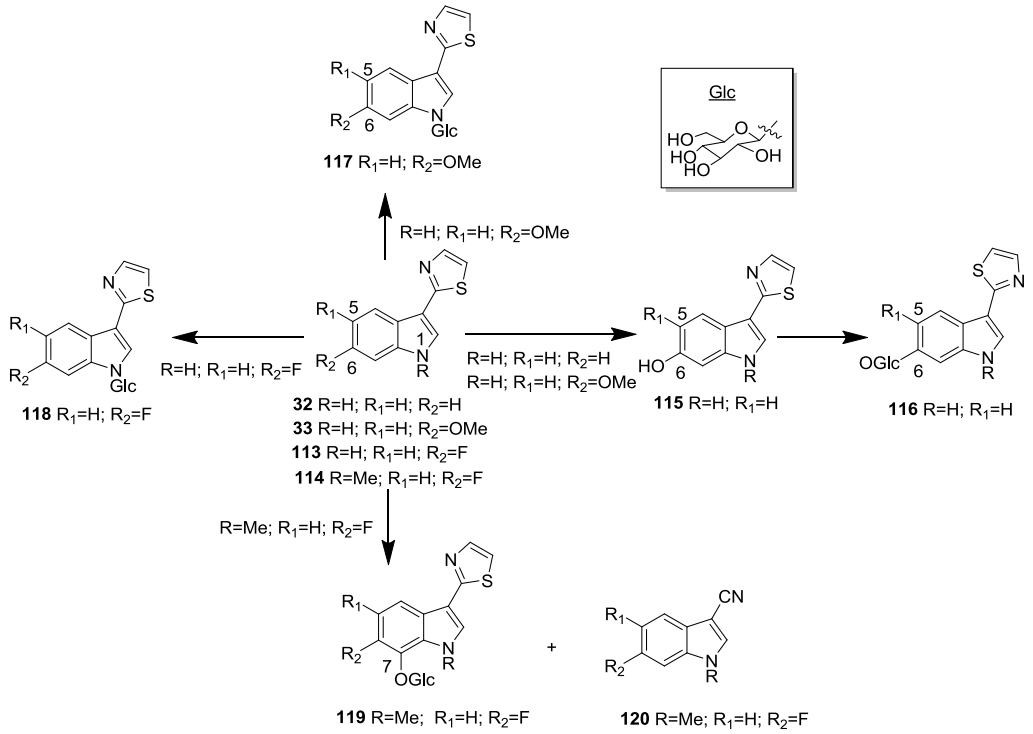
Scheme 1.11 Metabolism of spirobrassinins **27**, **28** and **105** by *Sclerotinia sclerotiorum* (Pedras and Hossain, 2006).

No glycosylation was observed in detoxifications of spirobrassinin (**27**) and analogs 1-methoxyspirobrassinin (**28**) and 1-methylspirobrassinin (**105**), compounds with lower inhibition effect against *S. sclerotiorum* (Scheme **1.11**) (Pedras and Hossain, 2006). Spirobrassinins **27** and **28** were metabolized to corresponding spirothiazolidinones **106** and **107** by *S. sclerotiorum*. Besides, a minor metabolite **108** was detected from metabolism of compound **28** by *S. sclerotiorum* (Pedras and Hossain, 2006). On the other hand, compound **105** was first metabolized to its demethylated product **27** through the intermediate **109**, which underwent loss of the hydroxymethyl group at N-1 to give compound **27**. In addition metabolite **110** was also detected from metabolism of compound **105** by *S. sclerotiorum* (Pedras and Hossain, 2006). *S. sclerotiorum* carried out both glycosylation and non-glycosylation modes of transformation in the case of a compound **111**, wherein the compound was hydroxylated at C-3 and glycosylated at N-1 to yield compound **112** (Scheme **1.12**) (Pedras and Hossain, 2007).



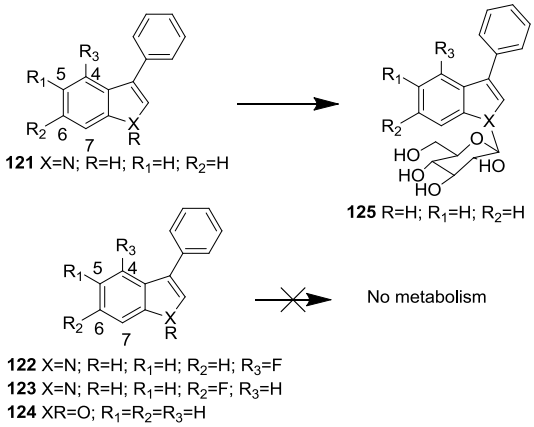
Scheme 1.12 Metabolism of isobrassinin (**111**) by *Sclerotinia sclerotiorum* (Pedras and Hossain, 2007).

The metabolism of camalexin (**32**) by several cruciferous fungi was investigated. Compound **32** was found to be metabolized by *R. solani* (Pedras and Khan, 1997), *S. sclerotiorum* (Pedras and Ahiahonu, 2002) and *B. cinerea* (Pedras, Hossain et al., 2011a). In addition, analogs of camalexin were screened for metabolism by *S. sclerotiorum* (Pedras and Ahiahonu, 2002) and *R. solani* (Pedras and Liu, 2004). These compounds were derivatives of camalexin (**32**) in which the indolyl hydrogens were replaced with atoms such as F, Me, OMe etc., or the thiazolyl ring was replaced with a phenyl ring (Pedras and Ahiahonu, 2002; Pedras and Liu, 2004; Pedras and Hossain, 2007). Camalexin (**32**) was metabolized by *S. sclerotiorum* to 6-oxy-(*O*-β-glucopyranosyl)camalexin (**116**) via 6-hydroxycamalexin (**115**) (Pedras and Ahiahonu, 2002). If C-6 of camalexin was blocked with OMe group, as in compound **33**, *S. sclerotiorum* metabolized compound **33** in the same manner to compound **32** via an oxidative demethylated intermediate 6-hydroxycamalexin (**115**). In addition, compound **33** was transformed to a minor metabolite **117** (Pedras and Ahiahonu, 2002). However, if C-6 of camalexin was blocked with F, the compound **113** was metabolized to *N*-glucosylated product **118** by *S. sclerotiorum* (Pedras and Ahiahonu, 2002). On the other hand, if C-6 and N-1 of camalexin analogs were blocked, as in compound **114**, the fungus glycosylated the compound **114** at C-7 (Scheme **1.13**). Compound **114** was transformed to metabolite **119** by *S. sclerotiorum*. In addition, compound **114** was also converted to compound **120** by *S. sclerotiorum* (Pedras and Ahiahonu, 2002). In general, *O*-glycosidation and *N*-glycosidation, but not C-glycosidation was observed among the detoxification reactions by *S. sclerotiorum*. In compounds where there are no NH/OH functionalities available for glycosylation, oxidation preceded the glycosylation.



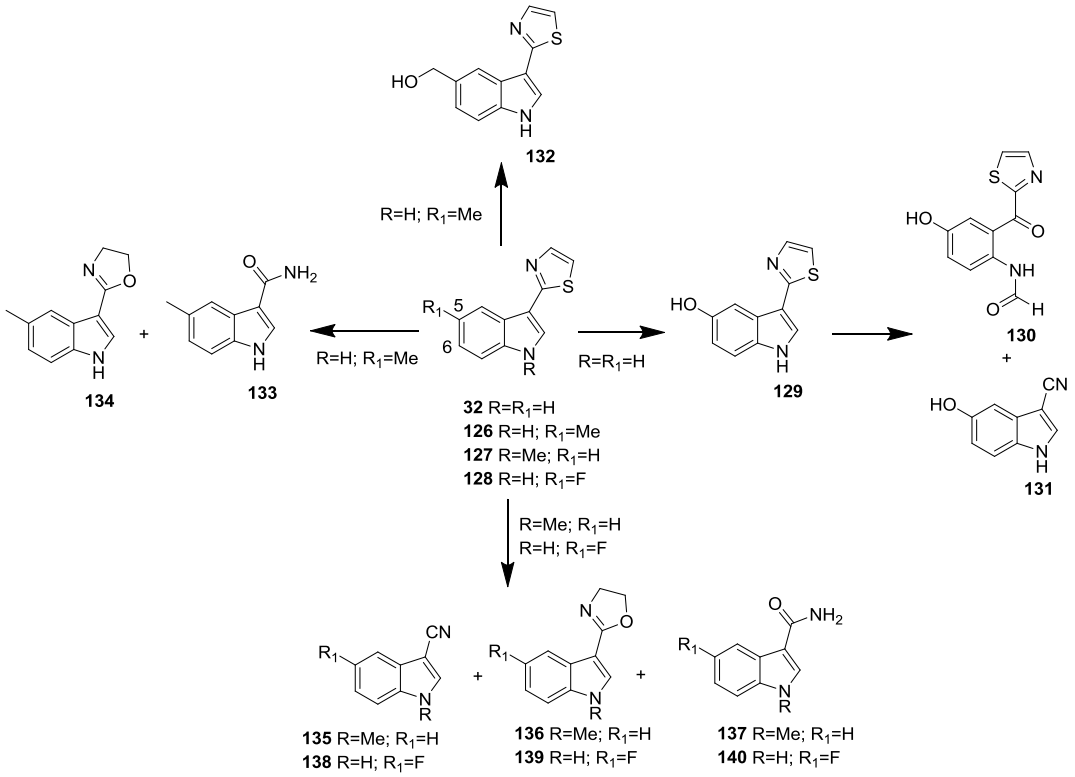
Scheme 1.13 Pathways of metabolism of camalexin (**32**) and analogs **33**, **113** and **114** by *Sclerotinia sclerotiorum* (Pedras and Ahiahonu, 2002)**.**

3-Phenylindole (**121**) was metabolized to the *N*-glycosylated compound **125** whereas, the analogs **122** and **123** were not metabolized by *S. sclerotiorum* (Pedras and Hossain, 2007). In addition, 3-phenylbenzofuran (**124**) in contrast to 3-phenylindole (**121**) was not metabolized by *S. sclerotiorum* (Scheme **1.14**) (Pedras and Hossain, 2007).



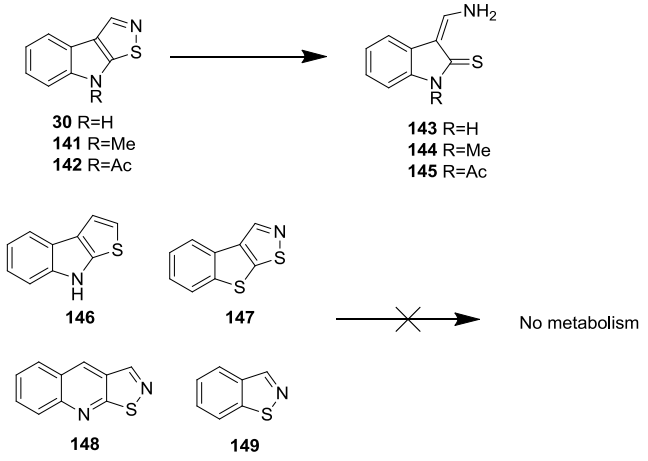
Scheme 1.14 Transformation of 3-phenylindole (**121**) and other compounds **122-124** by *Sclerotinia sclerotiorum* (Pedras and Hossain, 2007).

Camalexin analogs were metabolized by *R. solani* through various pathways (Pedras and Khan, 1997, 2000; Pedras and Liu, 2004). Oxidation of the indole ring is the predominant step in metabolism of compounds **32** and **126**. Camalexin (**32**) was metabolized to compound **129** through hydroxylation of C-5 (Pedras and Khan, 2000) whereas, compound **126**, with a Me group at position C-5 was metabolized to the hydroxymethyl compound **132**. Compound **129** was further metabolized to compounds **130** and **131** (Pedras and Liu, 2004). Compound **126** was metabolized by *R. solani* to the products **133** and **134** (Pedras and Liu, 2004). Compound **127** was metabolized to products **135-137** (Pedras and Liu, 2004) whereas, compound **128** was metabolized to products **138-140** (Pedras and Liu, 2004). Nitriles **135** and **138** were found to be the major metabolites in these transformations of compounds **127** and **128** by *R. solani* (Scheme **1.15**). These examples demonstrated that C-5 of the indole ring is the preferred site of transformation by *R. solani*. However, the site of transformation was switched to the thiazole ring if C-5 was blocked by F (Pedras and Liu, 2004).



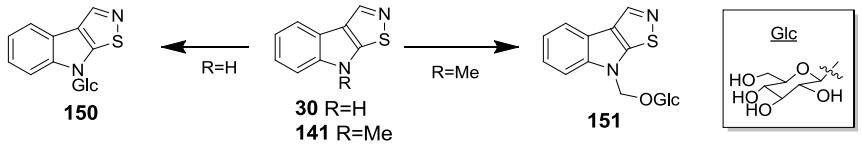
Scheme 1.15 Metabolism of camalexin (**32**) and analogs **126-128** by *Rhizoctinia solani* (Pedras and Khan, 2000; Pedras and Liu, 2004).

Brassilexin (**30**) was transformed by isolates of *L. maculans* virulent on canola (Pedras and Suchy, 2005) and mustard (Pedras and Snitynsky, 2010), and by *S. sclerotiorum* (Pedras and Hossain, 2006). The isothiazolyl ring was found to be the preferred site for transformation of brassilexin and its analogs by *L. maculans* (virulent on canola). Compound **30** was metabolized to 3-aminomethylene-indoline-2-thione (**143**) by *L. maculans* (virulent on canola) (Pedras and Suchy, 2005). Similarly, brassilexin derivatives **141** and **142** were metabolized to products **144** and **145** respectively. However, analog **146** and others **147**-**149**, in which the isothiazole ring system was fused with quinoline, phenyl or benzothiophene moieties were not metabolized by *L. maculans* (virulent on canola) (Scheme **1.16**) (Pedras and Suchy, 2006).



Scheme 1.16 Metabolism of brassilexin (**30**) and other compounds **141**, **142** and **146**-**149** by *Leptosphaeria maculans* (virulent on canola) (Pedras and Suchy, 2006).

Brassilexin (**30**) was metabolized to compound **150** by *S. sclerotiorum* through glycosylation at *N*-1, whereas compound **141** was first oxidized at the *N*-Me group and then glycosylated to product **151** (Scheme **1.17**) (Pedras and Hossain, 2006). Many phytoalexin analogs and related compounds were resistant to metabolism by cruciferous fungi (Appendix).



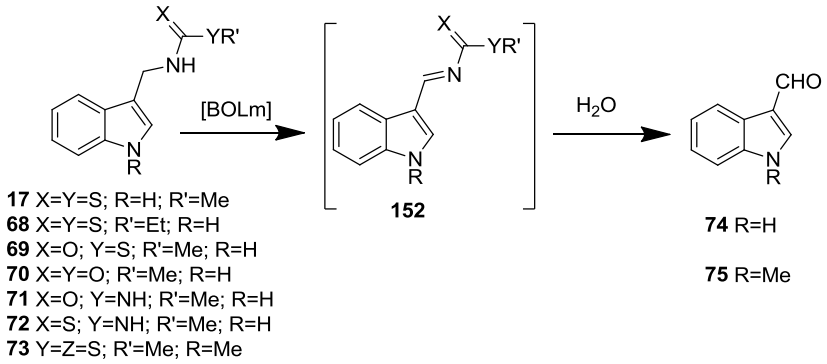
Scheme 1.17 Metabolism of brassilexin (**30**) and analog **141** by *Sclerotinia sclerotiorum* (Pedras and Hossain, 2006).

1.4.3 Enzymes and inhibitors of brassinin transformation by *Leptosphaeria maculans*

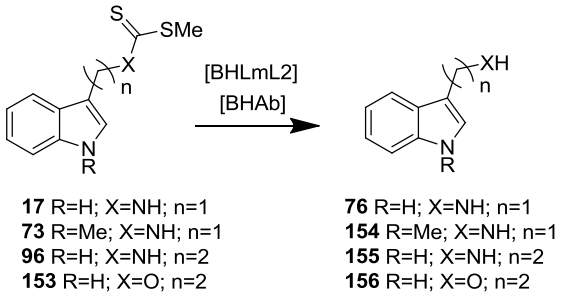
Four enzymes involved the detoxification of phytoalexin brassinin (**17**) were isolated from four fungal species (Pedras, Minic et al., 2008a; Pedras, Minic et al., 2009b; Sexton, Minic et al., 2009). Brassinin oxidase (BOLm) was the first cruciferous phytoalexin detoxifying enzyme isolated from *L. maculans* (virulent on canola) (Pedras, Minic et al., 2008a). BOLm has a molecular mass of 57 kDa and was found to be glycosylated up to 20% (Pedras, Minic et al., 2008a). BOLm catalyzed the transformation of brassinin (**17**) to indole-3-carboxaldehyde (**74**) through a potential intermediate **152**. Several analogs of brassinin were used to probe for substrate specificity of BOLm (Pedras, Jha et al., 2007c). Only compounds **17** and **68**-**73** acted as the substrates of BOLm (Scheme **1.18**). Based on these results, structural features essential for metabolism by BOLm were indicated (Pedras, Jha et al., 2007c). Key structural features identified for metabolism among the substrates were: the presence of methylene group bridging the 3-indolyl moiety with the side chain functional group containing the atoms X=O/S and Y=N/O/S; or presence of a free NH as part of the side chain functional groups such as carbonate, mono/dithiocarbamates; small alkyl groups such as methyl and ethyl (R=Me, Et) as part of the side chain (Pedras, Jha et al., 2007c).

Brassinin hydrolases BHLmL2 and BHAb were isolated from *L. maculans* (virulent on mustard) (Pedras, Minic et al., 2009b) and *A. brassicicola* (Pedras, Minic et al., 2009b), respectively. Both the enzymes transformed brassinin (**17**) to indolyl-3-methanamine (**76**). BHLmL2 was a tetrameric protein with a mass of 220 kDa, while BHAb was a

dimeric protein with a mass of 120 kDa. Both BHLmL2 and BHAb belonged to the family of amidases (Pedras, Minic et al., 2009b). Several analogs of brassinin were tested as probes for specificity and it was found that both BHs show high degree of substrate specificity. Only compounds **73**, **96** and **153** were metabolized by BHs in vitro (Scheme **1.19**) (Pedras, Minic et al., 2009b).



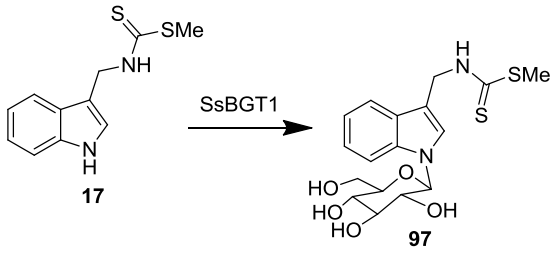
Scheme 1.18 Transformation of brassinin (**17**) and compounds **68**-**73** by brassinin oxidase (BOLm) from *Leptosphaeria maculans* (virulent on canola) (Pedras, Jha et al., 2007c).



Scheme 1.19 Transformation of brassinin (**17**) and compounds **73**, **96** and **153** by brassinin hydrolases from *Leptosphaeria maculans* (virulent on mustard) and *Alternaria brassicicola* (Pedras, Minic et al., 2009b)**.**

Another enzyme involved in the detoxification of brassinin (**17**) by *S. sclerotiorum* SsBGT1 was expressed in *Saccharomyces cerevisiae* and purified (Sexton, Minic et al., 2009). SsBGT1 converted brassinin (**17**) to 1-β-D-glucopyranosylbrassinin (**97**) (Scheme **1.20**). Two phytoalexins cyclobrassinin (**19**) and 6-methoxycamalexin (**33**),

and 3-phenylindole (**121**) were substrates of SsBGT1, but to a less extent relative to brassinin (**17**) (Sexton, Minic et al., 2009).



Scheme 1.20 Transformation of brassinin (**17**) by SsBGT1 cloned and expressed in *Saccharomyces cerevisiae* (Sexton, Minic et al., 2009).

L. maculans can effectively metabolize some cruciferous phytoalexins. As a result, infected plants are depleted of their chemical defenses and consequently, become more succeptible to the pathogens (Pedras, Yaya et al., 2011c; Pedras, 2008). On the contrary, if phytoalexins are resistant to fungal metabolism, they can protect plants from fungal attacks. In this context, brassinin (**17**) is of great importance because it is antifungal against *L. maculans* and is a precursor of other phytoalexins (Scheme **1.1**). However, compound **17** is rapidly metabolized by *L. maculans* and other pathogens (Appendix) through enzymatic transformations. Considering the advantages of the presence of brassinin (**17**) in infected plants, it was proposed that BOLm would be a reasonable metabolic target to control *L. maculans* (Pedras, 2011). Inhibition of crucial phytoalexin detoxification pathways in the crucifer pathogens may be an attractive strategy to control fungal diseases (Pedras, Jha et al., 2003). Pedras and co-workers introduced a concept of **p**hyto**al**exin **d**et**ox**ification **in**hibitor**s** (PALDOXINS) for disease control of specific fungal pathogens (Pedras, Jha et al., 2003). Paldoxins are desired to have low antifungal and cytotoxic activities to minimize their effects on cultivated ecosystems (Pedras, Minic et al., 2009c). Most importantly, paldoxins could enable plants to improve their own defense against pathogens.

Early work was directed towards the design and synthesis of substrate analog inhibitors. Isosteric replacement of the atoms N, C, S of the dithiocarbamate of

brassinin (**17**), and replacement of the indolyl moiety with phenyl and naphthyl moieties provided access to a library of about 80 compounds (Pedras and Jha, 2006). However, some of those compounds (Appendix) were metabolized when tested in fungal cultures of *L. maculans* and with purified BO (Scheme **1.19**). Compounds which resisted the metabolism were co-incubated with brassinin (**17**) in fungal cultures of which compounds **153** and **157-159** showed effect on the rates of brassinin detoxification in fungal cultures (Figure **1.6**) (Pedras and Jha, 2006).

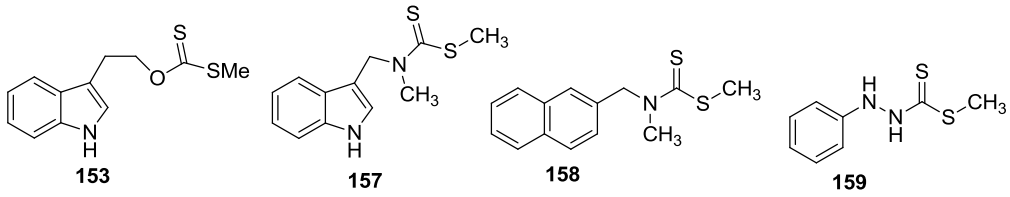


Figure 1.6 Structures of compounds **153**, and **157-159** that decreased the rate of brassinin transformation by *Leptosphaeria maculans* (virulent on canola) (Pedras and Jha, 2006).

After isolation of BOLm systematic enzymatic in vitro screening and evaluation of the inhibitors was carried out (Pedras, Jha et al., 2007c; Pedras, Minic et al., 2008a). However, none of the compounds designed based on the structure of brassinin (**17**) showed inhibition of BOLm activity (Pedras, Jha et al., 2007c). Early lead compounds of BOLm inhibitors were identified from a library of phytoalexins and synthetic compounds (Pedras, Jha et al., 2007c; Pedras, Minic et al., 2008a). Among the phytoalexins, cyclobrassinin (**19,** 37% at 0.30 mM), wasalexins (**25** and **26**, 14% at 0.30 mM**),** spirobrassinin (**27**, 34% at 0.30 mM), brassilexin (**30,** 16% at 0.30 mM) and camalexin (**32**, 53% at 0.30 mM) were identified as inhibitors of BOLm (Pedras, Jha et al., 2007c; Pedras, Minic et al., 2008a). Among the synthetic compounds, isobrassinin (**111**, 23% at 0.30 mM) and thiabendazole (**160**, 25% at 0.30 mM), a commercial fungicide, were also identified as inhibitors of BOLm (Figure **1.7**) (Pedras, Minic et al., 2008a).

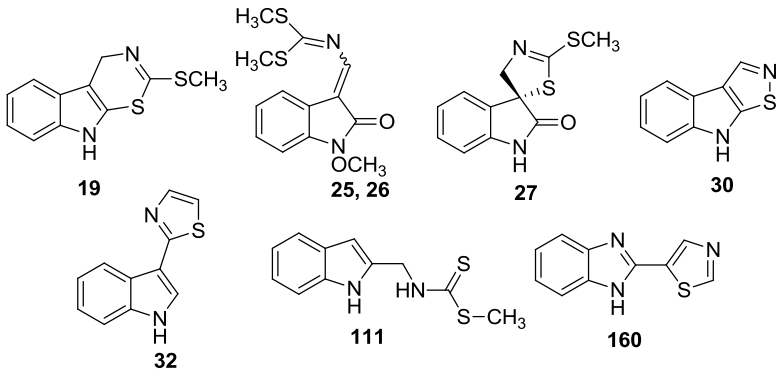


Figure 1.7 BOLm inhibitors: Phytoalexins **19**, **25-27**, **30**, **32** and synthetic compounds **111** and **160** (Pedras, Jha et al., 2007c; Pedras, Minic et al., 2008a).

Optimization studies of lead compounds **25**, **26**, **30** and **32** afforded better inhibitors of BOLm (Pedras, Minic et al., 2009c; Pedras, Minic et al., 2010a). Among the derivatives of brassilexin (**30),** 6-chlorobrassilexin (**162,** 93% at 0.30 mM) was discovered as the best inhibitor of BOLm to date (Pedras, Minic et al., 2010a). Other compounds from the series **161-166** showed significant inhibition. In general, 6-substituted brassilexins **162**, **164** and **166** were more inhibitory than their 5-substituted counterparts **161**, **163** and **165** (Pedras, Minic et al., 2010a). 5-Methoxycamalexin (**167,** 72% at 0.30 mM) was identified as the most active inhibitor of BOLm based on the structure of camalexin (**32**) (Pedras, Minic et al., 2009c). In general, 5-substituted camalexins **128** and **167** inhibited BOLm to a larger extent than their 6-substituted counterparts **33** and **113** (Pedras, Minic et al., 2009c). However, analogs of wasalexins, e.g. compound **172**, were no better than compounds **25** and **26** (Pedras, Minic et al., 2010a). Among the other compounds, 6-fluoro-3-phenylindole (**123**) and naphthylisothiazoles **168** and **170** were identified as inhibitors of BOLm (Figure **1.8**) (Pedras, Minic et al., 2009c). All the compounds that were screened for inhibition of BOLm along with their antifungal activities against *L. maculans* are summarized in the appendix section (Appendix **A1D**).

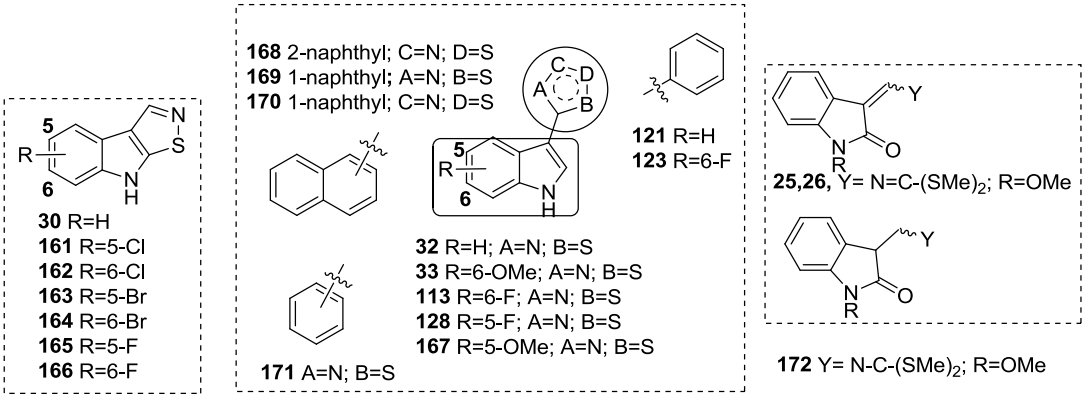


Figure 1.8 Phytoalexins **25**, **26**, **30**-**33** and related compounds **113**, **121**, **123**, **128** and **161**-**172** tested as inhibitors of BOLm (Pedras, Minic et al., 2009d; Pedras, Minic et al., 2010a).

Since BOLm activity was detected in leaves of blackleg-infected canola (Pedras, Minic et al., 2008a), it is expected that application of paldoxin-like compounds on the infected plants would control the infection of *L. maculans*. However, the utility of such compounds in the control of *L. maculans* is yet to be demonstrated.

The enzymatic targets of paldoxins are associated with secondary metabolic processes of fungal pathogens such as phytoalexin detoxification. By contrast, the traditional compounds used in crop protection (fungicides) target enzymes involved in fungal primary metabolism. In the following section, fungal enzyme inhibitors that are important in the control of fungal diseases fungicides, will be covered.

1.5 Crop protectants against pathogenic fungi

The first chemical used as plant protectant was copper sulfate. In 1807, Prevost used copper sulfate solutions to control the bunt of wheat crop (Russell, 2005; Klittich, 2008). Later in 1885, the Bordeaux mixture, a mixture of copper sulfate and lime, was invented as first foliar fungicide to treat downy mildew on grapevines and others

(McCallan, 1939; Russell, 2005; Klittich, 2008). In the 1930s, dithiocarbamates came into light as broad spectrum fungicides and they were effective in the treatments of blight and leaf spot diseases of vegetables and fruits (Houeto, Bindoula et al., 1995; Russell, 2005). Examples of dithiocarbamate fungicides include thiram (**173**), maneb (**174**), zineb (**175**) and mancozeb (**176**) (Gullino, Tinivella et al., 2010). Mancozeb (**176**), a 1:10 mixture of compounds **174** and **175** was developed in 1961 and is still a widely used fungicide (Russell, 2005; Gullino, Tinivella et al., 2010). Discovery of the systemic fungicide carboxin (**177**) for cereal seed treatments and later, benomyl (**178**) as broad spectrum foliar fungicides (Figure **1.9**) opened a new era in the chemical control of fungal diseases in the 1970s (Russell, 2005). Research from many agrochemical industries like Bayer, Syngenta and Dupont led to significant advancements and now there are several commercial fungicides available as plant protectants (Russell, 2005; Klittich, 2008). Further studies on the mode of action of fungicides and the development of resistance against these compounds in various organisms effectively accelerated research in this area. Currently, a team of experts from FRAC (Fungicide Resistance Action Committee) guides and monitors all the commercial fungicidal crop protectants (Source: www.frac.info).

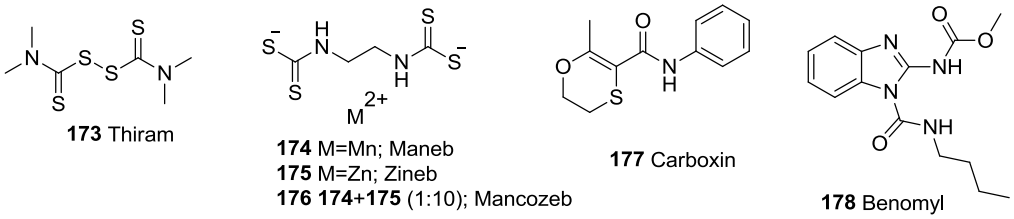


Figure 1.9 Selected early fungicides used in the treatment of fungal diseases dithiocarbamates **173-176**, carboxin (**177**) and benomyl (**178**) (Russell, 2005; Klittich, 2008; Gullino, Tinivella et al., 2010).

Some of the modern fungicides are inhibitors of metabolic processes occurring in fungi, including those involved in production of energy (e.g. fungal respiration) and biosynthesis of key metabolites (ergosterol, nucleic acids, methionine, melanin etc.) (Sisler, 1969; Leroux, 2003; Yamaguchi and Fujimura, 2005) (Source: www.frac.info).

Of these, fungicides acting on ergosterol biosynthesis and on the electron transport chain in mitochondria constitute the major portion of the fungicide sales (Casida, 2009). In this section, commercial fungicides that are known to inhibit specific fungal enzymes and compounds that are inhibitors of fungal enzymes and potential crop protectants against fungal diseases will be discussed.

1.5.1 Inhibitors of fungal respiration

In eucaryotes, respiration occurs in mitochondria of the cell. At this site, electrons are transferred from NADH to molecular oxygen. This redox process is compensated by proton translocation across the inner membrane of mitochondria and eventually, the proton gradient drives ATP synthesis and other processes (Saraste, 1999). This complex respiratory chain involves four membrane-bound respiratory enzyme complexes I-IV and electron carriers such as cytochrome b, c and ubiquinone (Saraste, 1999; Yamaguchi and Fujimura, 2005).

The four enzyme complexes are located sequencially within the inner membrane of mitochondria. At complex I (NADH:ubiquinone oxidoreductase), electrons are transferred from NADH to ubiquinone (Saraste, 1999). At complex III (cytochrome bc1), electrons are transferred from ubiquinol to cytochrome c and finally, at complex IV electrons are transferred from cytochrome c to molecular oxygen (Saraste, 1999; Yamaguchi and Fujimura, 2005). Complexes I, III and IV are directly associated with electron transfer, whereas, complex II assists the process by pumping two electrons into the ubiquinone pool (Avenot and Michailides, 2010; Omura and Shiomi, 2007).

Many of the fungicidal compounds work by inhibiting respiratory chain enzymes. Due to the importance of this process in fungal metabolism, fungicides that inhibit enzymes involved in respiration are abundant. Especially, inhibitors of complex III found major applications in fungicide development (Knight, Anthony et al., 1997; Sauter, Steglich et al., 1999).

1.5.1.1 Complex I inhibitors

Inhibitors of complex I with fungicidal activity are few. By far, diflumetorim (**179**), a pyridinamine, introduced by Yamanaka and co-workers, is the only commercial fungicide which is an inhibitor of complex I (Source: www.frac.info). However, this target has been of interest for several agrochemical industries. Recently, a series of novel aryloxylepidin analogs of general structures **182** and **183** were synthesized and used in lead-optimization studies. These compounds were designed as hybrid structures of fungicides fenazaquin (**180**) and quinoxyfen (**181**), an inhibitor of complex I and moderately active fungicide (Figure **1.10**) (Kirby, Daeuble et al., 2001). Among the series of aryloxylepidines **182** and **183**, only 8-halo (F, Cl) substituted compounds showed moderate inhibition of complex I and moderate fungicidal activities against *Ustilago maydis* (Kirby, Daeuble et al., 2001).

Besides, natural products such as ajudazol (**184**), myxalamid (**185**), aurachin (**186**), phenoxan (**187**), pyrrolnitrin (**188**) and thiangazole (**189**) isolated from myxobacteria are known to be antifungal and inhibit fungal respiratory enzymes of complex I (Figure **1.11**) (Degli Esposti, 1994, 1998; Weissman and Mueller, 2010). Based on the analysis of inhibitors of complex I, it was determined that inhibitors could bind to three different sites in the complex I. But all of the sites shared a major common portion for binding (Okun, Lummen et al., 1999).

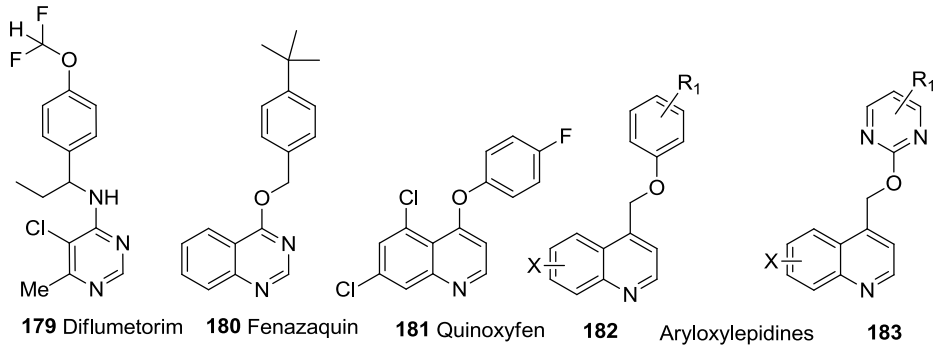


Figure 1.10 Inhibitors of complex I; diflumetorim (**179**), fenazaquin (**180**), quinoxyfen (**181**), and aryloxylepidines **182** and **183** used in SAR studies (Kirby, Daeuble et al., 2001).

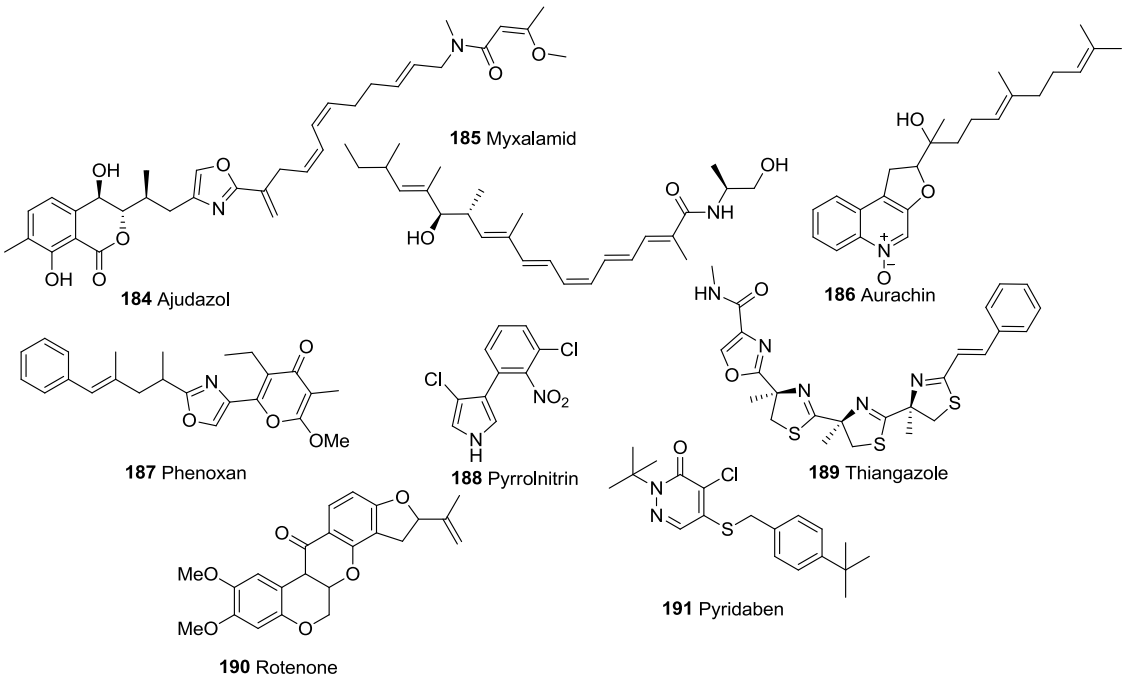


Figure 1.11 Inhibitors of complex I, natural products **184-191** (Degli Esposti, 1998; Hollingworth, Ahammadsahib et al., 1994; Weissman and Mueller, 2010).

Many of the complex I inhibitors do not possess fungicidal or antifungal activities. Inhibitors of mitochondrial electron transport complex I have additional biological activities (Degli Esposti, 1998). For example, rotenone (**190**) and pyridaben (**191**) have displayed insecticidal and acaricidal activity (Hollingworth, Ahammadsahib et al., 1994).

1.5.1.2 Complex II inhibitors

Complex II is a family associated with two integral membrane proteins succinate dehydrogenase and succinate:ubiquinone oxidoreductase (Saraste, 1999; Horsefield, Yankovskaya et al., 2006). The natural products harzianopyridone (**192**), isolated from culture broth of *Trichoderma harzianum*, and atpenins A4 (**193**), A5 (**194**) from broth of *Pencillium* sp. are known to inhibit succinate:ubiquinone reductase (complex II) (Figure **1.12**) (Miyadera, Shiomi et al., 2003; Omura and Shiomi, 2007). Inhibition by

these compounds is known to be specific to complex II, but not complex I (Miyadera, Shiomi et al., 2003). IC_{50} values of complex II inhibition by atpenin A5 (**194**) was 300 fold lower than that of carboxin (**177**), a commercial fungicide (Omura and Shiomi, 2007). However, compounds **192**-**194** showed low fungicidal activity compared to that of the fungicide carboxin (**177**). On the other hand, natural products ubiquinone (**195**), dinitrophenol (**196**) and 2-theonyltrifluoroacetate (**197**) inhibited complex II activity but did not possess any fungicidal properties (Horsefield, Yankovskaya et al., 2006).

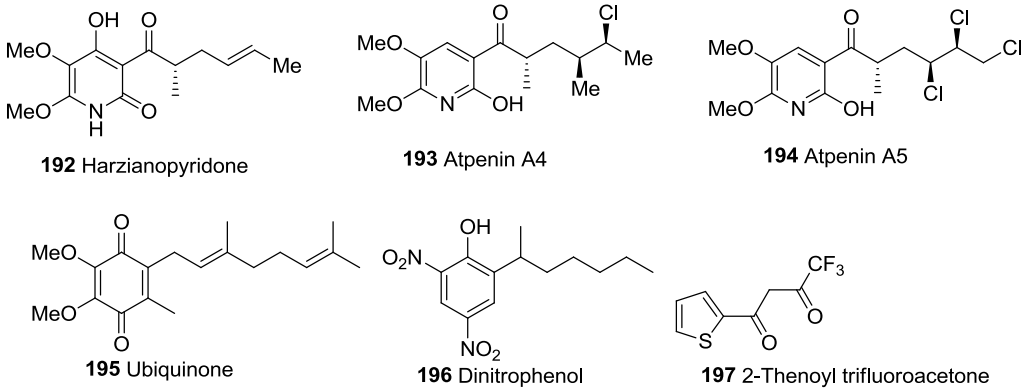


Figure 1.12 Inhibitors of complex II, natural products **192**-**197** (Horsefield, Yankovskaya et al., 2006; Miyadera, Shiomi et al., 2003; Omura and Shiomi, 2007).

Several classes of compounds have been synthesized and studied for inhibition of complex II activity. Important findings of structure-activity relationship (SAR) studies came from White et al., based on the lead structure carboxin (**177**) (White, 1971, 1987, 1988, 1989, White and Georgopoulos, 1986; White, Phillips et al., 1986; White and Thorn, 1975, 1980; White, Thorn et al., 1978). Carboxin (**177**) was first identified as a systemic fungicide against *Ureomycis phaseoli*, *Ustilago nuda* and *R. solani* (Vonschme.B and Kulka, 1966). White and co-workers identified the basic structure required for the enzyme inhibition activity (White, 1988) and exploited the toxophore in the synthesis of classes of compounds such as oxathiin carboxamides of general structure **198** (White, 1971), thiophene carboxamides of general structure **199** (White and Georgopoulos, 1986), pyrazole carboxanilides of general structure **200** (White, Phillips et al., 1986), substituted benzanilides of general structure **201** (White, 1987) and furan carboxamides of general structure **202** (Figure **1.13**) (White, 1988).

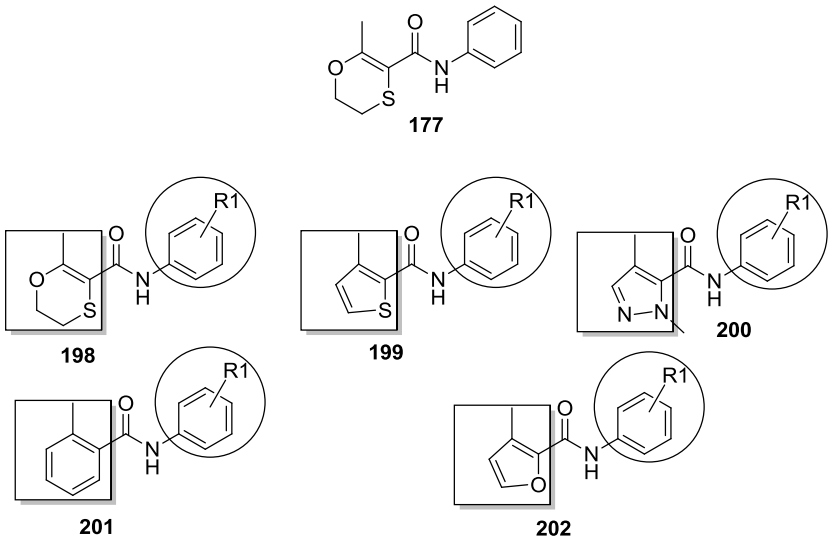


Figure 1.13 Complex II inhibitors designed based on structure of lead carboxin (**177**); oxathiin carboxamides **198**, thiophene carboxamides **199**, pyrazole carboxanilides **200**, substituted benzanilides **201**, furan carboxamides **202** (White, 1971, 1987, 1988, 1989, White and Georgopoulos, 1986; White, Phillips et al., 1986; White and Thorn, 1975, 1980; White, Thorn et al., 1978).

Selby, Hughes et al. (2010) designed and synthesized inhibitors of general structures **203** and **204** based on the lead natural product atpenin A (**193**) (Figure **1.14**). Pyridones, analogs with general structure **203**, were found to be moderate inhibitors of complex II, while dihyropyridines, analogs with general structure **204**, were more potent complex II inhibitors compared to atpenin A (**300**). Especially, compounds resulting from substitution of methyl-t-butyl side chain in the structure **311** inhibited complex II better than atpenin A (**193**) (Selby, Hughes et al., 2010).

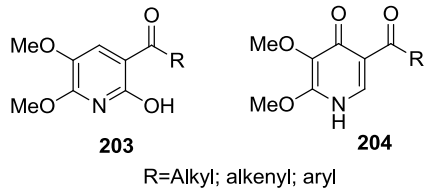


Figure 1.14 Complex II inhibitors **203** and **204** designed based on the lead structure atpenin A (**193**) (Selby, Hughes et al., 2010).

1.5.1.3 Complex III inhibitors

Complex III catalyzes the electron transfer from ubiquinol to cytochrome c. This process is coupled to translocation of the protons through the Q-cycle at two ubiquinone redox sites Q_i and Q_0 (Saraste, 1999). Complex III inhibitors include strobilurins, which are derivatives of the natural compounds strobilurin A (**205**) and oudemansin A (**215**) isolated from fungi (Bartlett, Clogh et al., 2001). Strobilurin A (**205**) was first isolated from basidiomycete *Strobilurus tenacellus* by Anke and co-workers (Anke, Oberwinkler et al., 1977). Oudemansin A (**215**) was first identified by Anke and co-workers in 1978 (Anke, Hecht et al., 1979), and later many other strobilurins **206**-**214** have been isolated (Figure **1.15**) (Sauter, Steglich et al., 1999). Strobilurin fungicides bind to the Q_0 site of cytochrome b and block the supply of electrons to cytochrome c. This blockage in turn affects the production of ATP (Degli Esposti, Crimi et al., 1994).

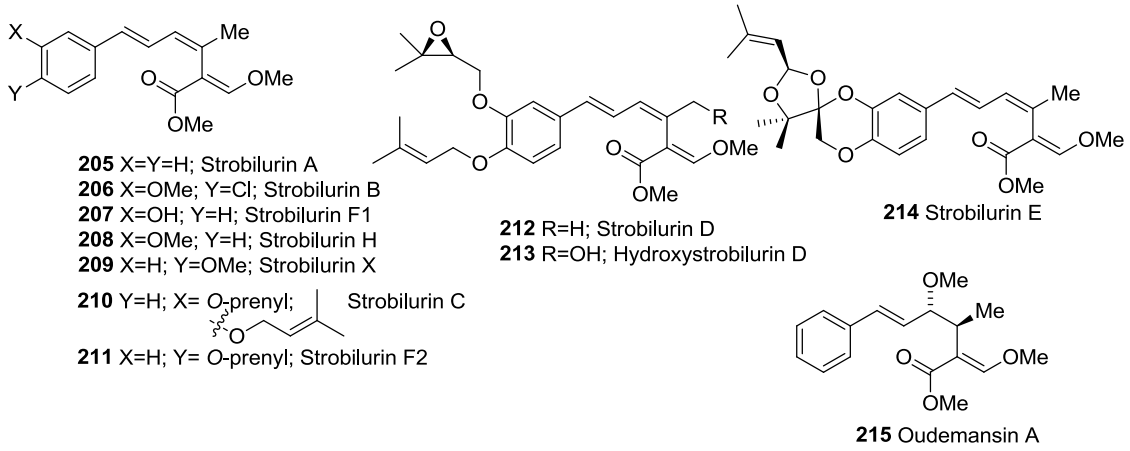


Figure 1.15 Complex III inhibitors, natural products strobilurin A (**205**), oudemansin A (**215**) and others **206**-**214** (Bartlett, Clogh et al., 2001; Sauter, Steglich et al., 1999).

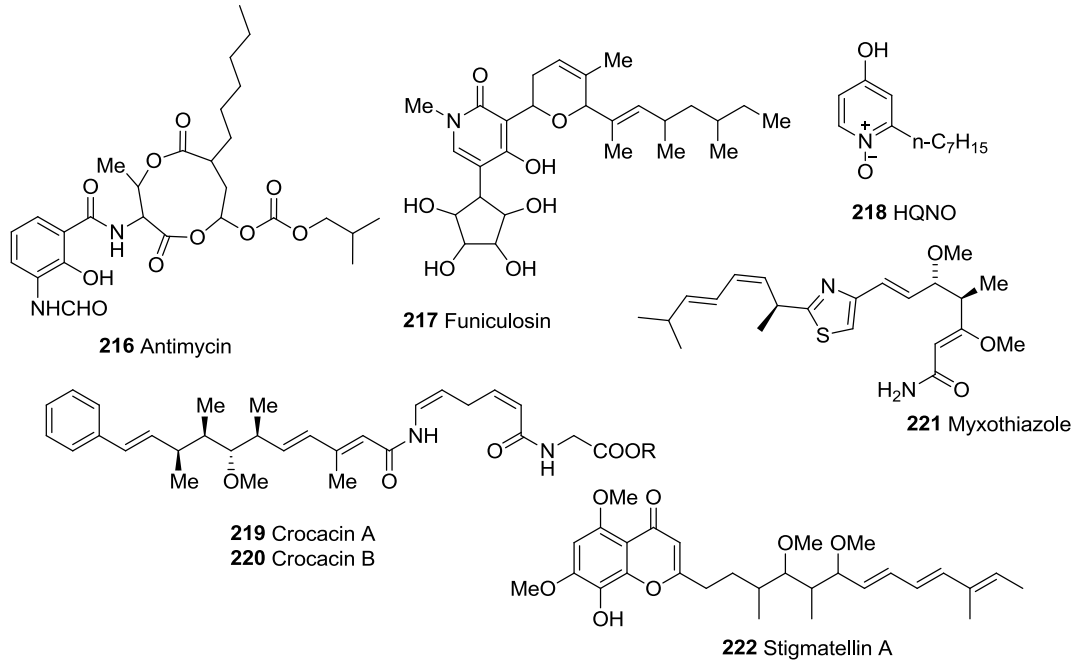


Figure 1.16 Complex III inhibitors, other natural products **216**-**222** (Rich, 1996; Rieske, 1980).

Other natural product inhibitors of complex III are shown in figure **1.16**. Compounds antimycin A (**216**), funiculosin (**217**) and 4-hydroxy-quinoline-*N*-oxide (HQNO, **218**) are known to bind to the Qi site (Rieske, 1980). Crocacins **219** and **220** were found to be inhibitors of complex III and recently analogs of crocacins were prepared for optimizing the complex III inhibition of the natural products (Crowley, Berry et al., 2008). The natural products of microbial origin myxothiazole (**221**) (Weissman and Mueller, 2010) and stigmatellin (**222**) (Vonjagow and Ohnishi, 1985) bind to Qo site of cytochrome bc1 complex.

Research in the area of fungicidal development was revolutionized with the invention of the strobilurin fungicides (Nising, Hillebrand et al., 2011). The natural strobilurins were unstable under sunlight and thus, were not effective for crop disease treatments. Many agrochemical industries like Syngenta and BASF were interested in the development of these compounds because of their novel mode of action. In these studies, the optimized structure **223** obtained from natural products **205** and **215** was

used (Nising, Hillebrand et al., 2011). This enol ether compound **223** contained three potential parts for further modification, i.e. (a) the toxophore group: methyl (E)-3-methoxy-2-(5-phenylpenta-2,4-dienyl) acrylate, (b) a bridge and (c) a side chain (Sauter, Steglich et al., 1999). Attempts to modify part A led to Kresoxym methyl (**224**), which was a basis for later development of other strobilurins **227**-**231**. These synthetic strobilurins had the toxophore derived from oximino acetate moiety and included changes in the side chain part C (Sauter, Steglich et al., 1999). Modification of general structure **223** by changing the sidechain, but not the toxophore and the bridge, led to the discovery of the fungicides **225**-**226** (Bartlett, Clogh et al., 2002; Sauter and Steglich et al., 1999). Several thousands of compounds were synthesized and were tested for the fungicidal activities. The major modifications and the gradual evolution of strobilurins is shown in figure **1.17**.

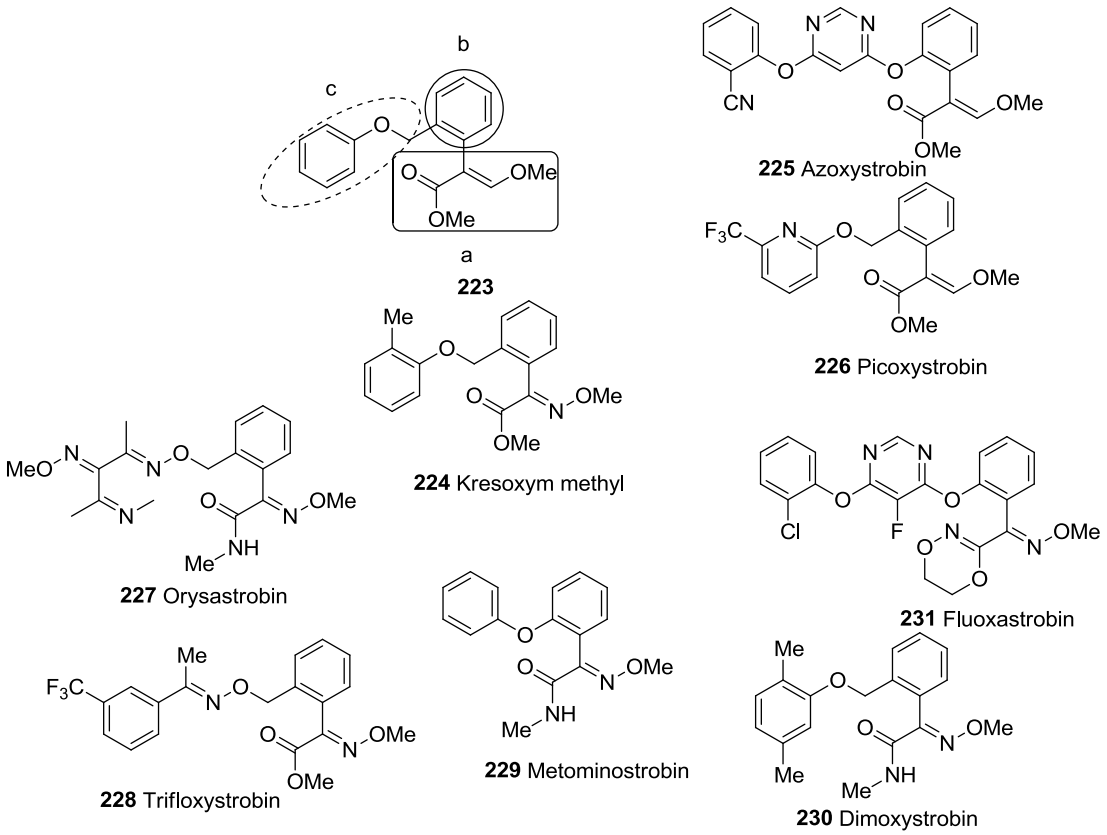


Figure 1.17 Evolution of strobilurin fungicides **223**-**231** (Bartlett, Clogh et al., 2002; Nising, Hillebrand et al., 2011; Sauter, Steglich et al., 1999).

On the other hand, researchers at DuPont developed oxazolidinone fungicides based on lead structure **232** from Geffken's lab. SAR studies based on the lead structure **232** led to identification of the fungicide famoxadone (**233**) (Sternberg, Geffken et al., 2001). About seven hundred ring analogs falling into general structures **234**-**238** were prepared (Figure **1.18**) from the lead structure **232**, of which several fungicidal compounds were identified. Eventually, famoxadone (**233**) was launched as the first potent fungicide among this class of compounds (Sternberg, Geffken et al., 2001). Wang et al. recently synthesized more analogs of general structures **239** and **240** based on the structure of famoxadone (**233**) and identified a more potent inhibitor from the compounds of general structure **240** (Wang, Li et al., 2011). Later, fenamidone (**241**) was identified to be active against *Plasmophora viticola* (Genix, Guesnet et al., 2003) and developed as a fungicide. Compound **241** has structural features similar to the ring analogs of general structure **237**. Optimization of the activities of compound **241** was attempted by Genix. Several ring analogs resembling the general structures **234**-**238** were prepared. It was found that compound **241** but not its analogs inhibited complex III (Genix and Villier, 2003). In particular, only *S*- but not the *R*-enantiomer of compound **241** inhibited the enzymatic activity (Genix and Villier, 2003).

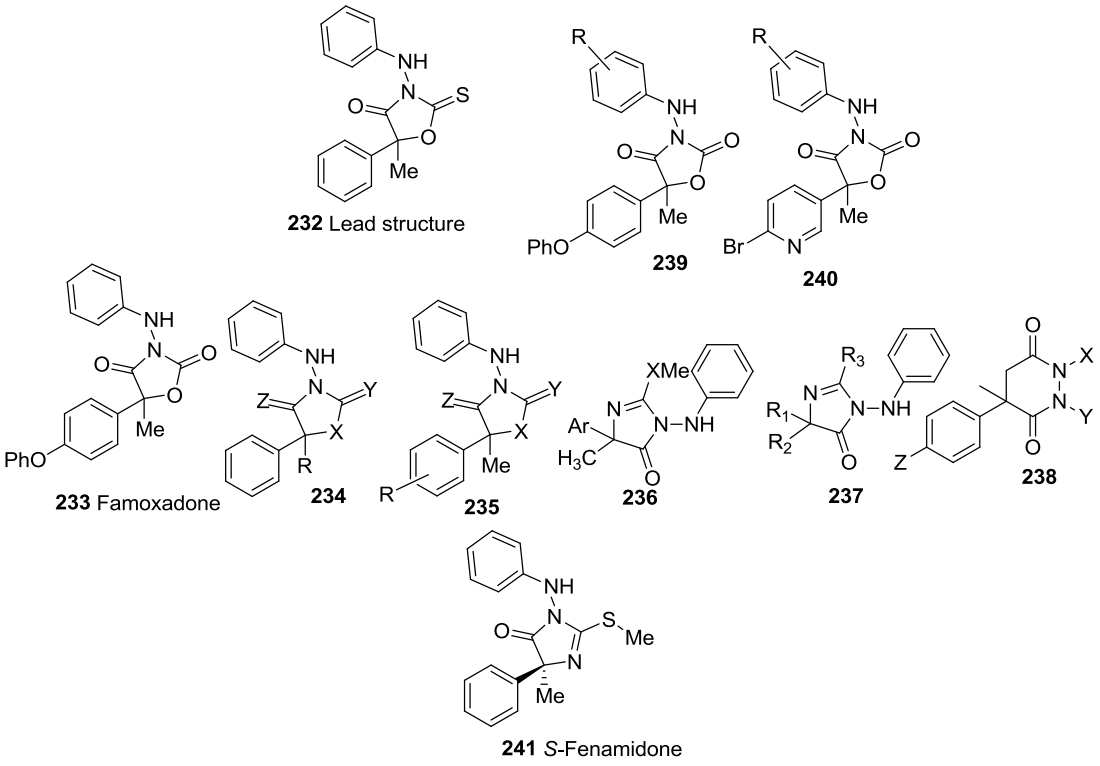


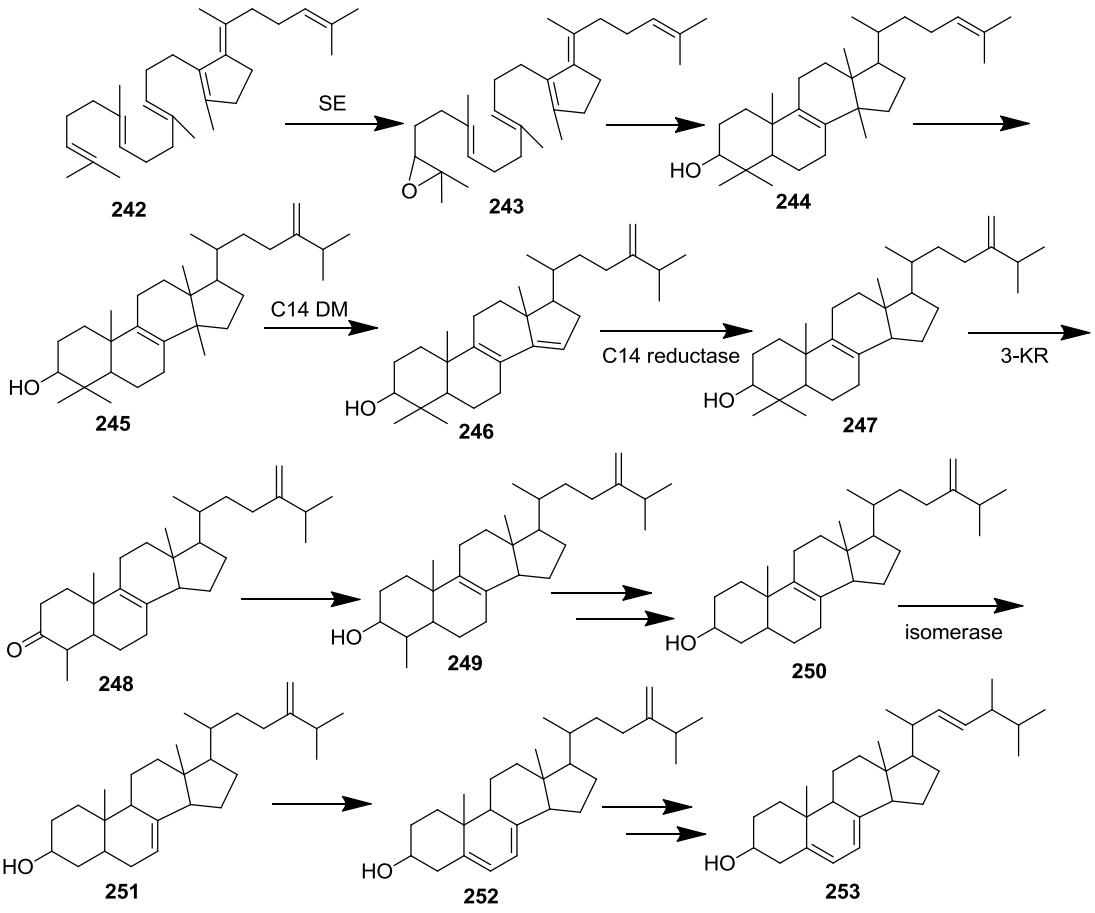
Figure 1.18 Inhibitors of complex III, azolones **232-241** (Genix, Guesnet et al., 2003; Sternberg, Geffken et al., 2001; Wang, Li et al., 2011).

Strobilurins display very poor specificity towards inhibition of bc1 complex among different species. Inhibition activitiy tests carried out in mitochondrial preparations of different species such as yeast, *Botrytis*, maize, house fly and rat with the help of 14 strobilurin analogs along with myxothiazole suggested similar activity patterns (Roehl and Sauter, 1994).

1.5.2 Inhibitors of ergosterol biosynthesis

Sterols are important components of cell membranes of fungi. The major sterol found among fungi is ergosterol. Mercer (1984, 1981) described the ergosterol biosynthesis in two parts namely, pre-epoxysqualene and post-squalene stages. The enzymatic steps in the pre-squalene stage are common for sterol synthesis in animals, higher plants and

fungi. In order to maintain the selectivity in inhibition of only ergosterol biosynthesis, researchers were interested in the enzymes involved in the post-squalene stage (Mercer, 1991). The steps involved in the synthesis of ergosterol (**253**) starting from squalene (**242**) via important intermediates **243**-**252** and the relevant enzymes are summarized in scheme **1.21** (Leroux, Fritz et al., 2002; Mercer, 1991).



Scheme 1.21 Post-squalene pathway of ergosterol (**253**) biosynthesis; enzymes used in the description: SE (squalene epoxidase), C14 DM (C14 demethylase), C14 reductase, 3 KR (3 keto reductase) and isomerase (Leroux, Fritz et al., 2002; Mercer, 1991).

Many of the fungicides interfere with ergosterol biosynthesis by inhibiting the key enzymes involved in the biosynthesis (Mercer, 1991; Leroux, 2003; Casida, 2009). Based on the target enzyme inhibition, sterol biosynthesis inhibitors could be of

different types: C14 demethylase inhibitors (C14 DMI; azoles), C14 reductase inhibitors, 3-keto reductase inhibitors (3-KR), Δ^8 - Δ^7 isomerase inhibitors (morpholines) and squalene epoxidase inhibitors (Mercer, 1991).

1.5.2.1 Inhibitors of demethylase (DMI)

A series of imidazoles, triazoles (collectively called azoles), pyridines, pyrimidines and piperazines are known to inhibit demethylation of the substrate dihydrolanosterol (**245**) (Baldwin, 1983). This conversion is catalyzed by the enzyme 14α-demethylase (Scheme **1.21**) (Mercer, 1984). During the 1970's the fungicides prochloraz (**254**), imizalil (**255**), propiconazole (**256**), prothioconazole (**257**) and triadimefon (**258**) were introduced. These compounds showed a broad spectrum of fungicidal activity on many pathogens of cereals, fruits, peanuts and coffee (Baldwin, 1983). Yanosaka et al. synthesized the novel carboxylate derivatives of imidazoles **259**-**261** shown in figure **1.19** (Yanosaka, Imai et al., 1991). Compounds **260** and **261** with methoxy and ethoxy substituents inhibited the growth of *B. cinerea* which was reflected in the amount of ergosterol synthesized. These compounds also showed potent inhibition of 14α-demethylase activity (Yanosaka, Imai et al., 1991). Recently, Arnoldi et al. synthesized novel compounds of general structures **263** and **264** with fungicidal activity based on tetraconazole (**262**). However, these compounds did not have improved activities but had comparable fungicidal activities with respect to tetraconazole (**262**). All the compounds acted as inhibtors of 14α-demethylase (Arnoldi, Carzaniga et al., 2000). Triforin (**265**), another systemic fungicide with mode of action of 14α-demethylase inhibition belongs to piperazine group of compounds (Figure **1.19**). Compound **265** was found to be active on *Aspergillus fumigatus* (Sherald and Sisler, 1975).

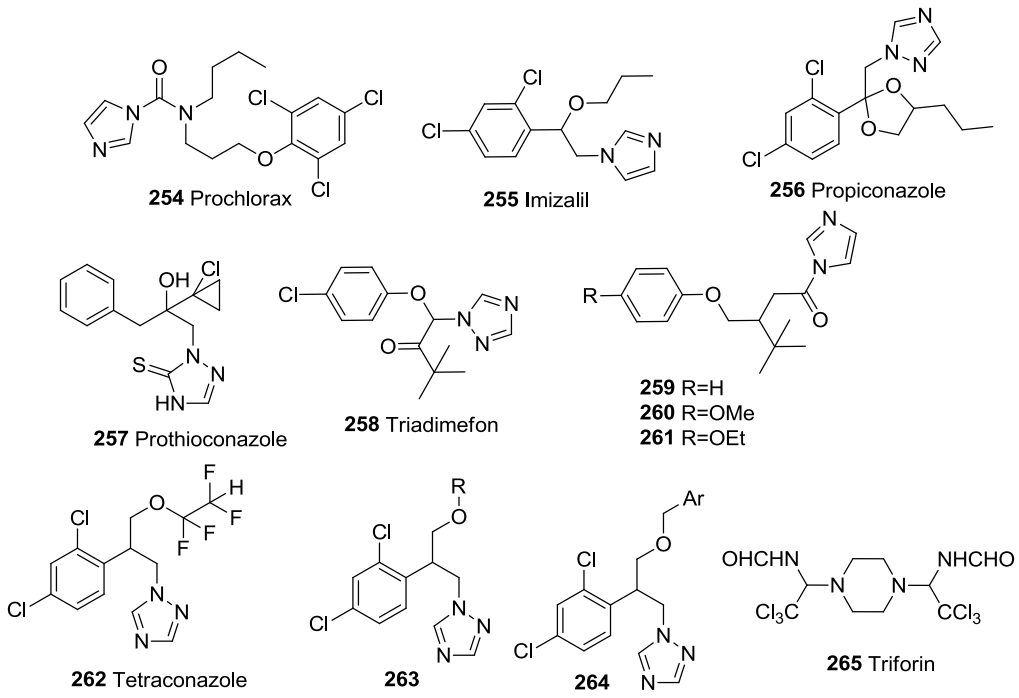
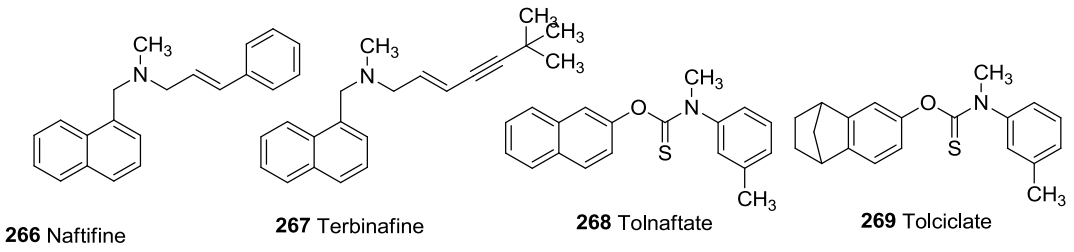


Figure 1.19 Structures of inhibitors of ergosterol C-14 demethylase **254-265** (Baldwin, 1983; Yanosaka, Imai et al., 1991; Arnoldi, Carzaniga et al., 2000).

1.5.2.2 Inhibitors of squalene epoxidase

Squalene epoxidase inhibitors inhibit the enzyme involved in the epoxidation of squalene (**242**) to form compound **243** (Scheme **1.21**). Allyl amines are an important class of compounds among squalene epoxidase inhibitors (Petranyi, Ryder et al., 1984). Naftifine (**266**) was one of the early allyl amine inhibitors of squalene epoxidase and an effective antimycotic (Georgopoulos, Petranyi et al., 1981). Structural modification of compound **266** led to development of the potent antifungal compound terbinafine (**267**). Compound **267** was determined as a specific inhibitor of fungal squalene epoxidase (Petranyi, Ryder et al., 1984). Terbinafine (**267**) is known to have potent fungicidal activities against many pathogens because of its inhibition of squalene epoxidase. It was found out experimentally that fungi fed with compound **267** accumulated squalene (**242**), and failed to biosynthesize ergosterol (**253**). Tolnafthate (**268**) and tolciclate (**269**) were later identified as inhibitors of ergosterol biosynthesis

(Figure **1.20**) (Ryder, Frank et al., 1986). These thiocarbamate compounds inhibited the squalene epoxidase activity from *Candida albicans* (Ryder, Frank et al., 1986). A good correlation between ergosterol biosynthesis inhibition by compounds **268** and **269** and their antifungal activity against *C. albicans* was observed (Ryder, Frank et al., 1986).



266 Naftifine **267** Terbinafine **268** Tolnaftate **269** Tolciclate

Figure 1.20 Inhibitors of squalene epoxidase **266**-**269** (Georgopoulos, Petranyi et al., 1981; Petranyi, Ryder et al., 1984; Ryder, Frank et al., 1986).

1.5.2.3 Inhibitors of Δ14 reductase and Δ8 - Δ7 isomerase

Δ14 - Reductase catalyzes the conversion of compound **246** to **247** and Δ8 - Δ7 isomerase converts compound **250** to **251** in the biosynthetic pathway leading to ergosterol (**253**) (Scheme **1.21**). Morpholines, 3-phenylpropylamines and spiroketalamines are important classes of fungicides known to inhibit these two enzymes (Leroux, Fritz et al., 2002).

Morpholines with potent fungicidal properties were identified in 1960s (Konig, Pommer et al., 1965). Substitution of the N-atom of general structure **270** with an aliphatic carbon chain with 10-12 carbon atoms resulted in improved fungicidal activities as in tridemorph (**271**) (Konig, Pommer et al., 1965). It is also known that morpholines and their structural analogs inhibit sterol biosynthesis in higher plants (Rahier, Schmitt et al., 1986). Further optimization of morpholine-based analogs with general structure **270** led to the identification of the more potent fungicides fenpropiomorph (**272**) and fenpropidin (**273**). Compound **272** contains a phenylpropyl

unit attached to morpholine and in the other compound **273**, the morpholine ring was replaced with piperidine ring (Figure **1.21**). Both the compounds **272** and **273** showed fungicidal properties against powdery mildews (Himmele and Pommer, 1980). However, the in vitro assays using enzymes isolated from *S. cerevisiae* demonstrated that tridemorph (**271**) was a strong inhibitor of Δ8 - Δ7 isomerase, whereas compounds **272** and **273** showed inhibition of both Δ14 reductase and Δ8 - Δ7 isomerase (Baloch and Mercer, 1987). Compounds **272** and **273** also were active against *Microdochium nivale* and also inhibited Δ8 - Δ7 isomerase activity in cell free assays (Debieu, Bach et al., 2000).

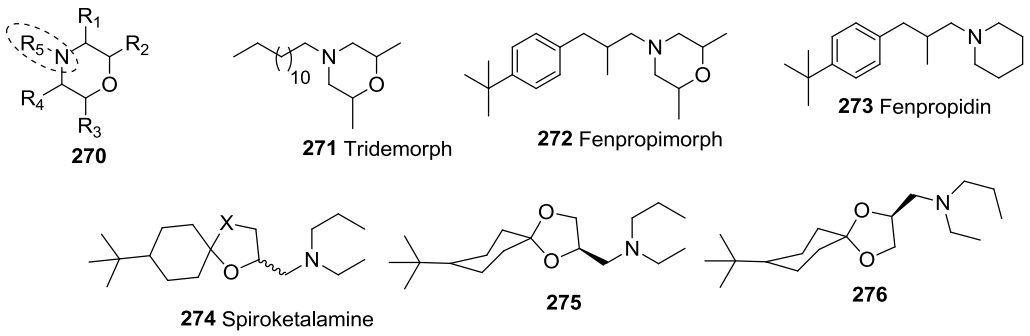


Figure 1.21 Inhibitors of sterol biosynthesis: morpholines **270** and related fungicides **271**-**273** and spiroxamines **274**-**276** (Baloch and Mercer, 1987; Kraemer, Berg et al., 1999; Debieu, Bach et al., 2000).

Later, a class of compounds representing the general structure of spiroketalamine **274** was developed. Spiroxamine or KWG 4168 (**274**), the most active fungicide from this class, had four isomers, of which compounds **275** and **276** were biologically active (Figure **1.21**). Compound **274** showed inhibition of Δ14 reductase (Kraemer, Berg et al., 1999) and was very effective, with a broad spectrum of activity. It showed good activity against cereal rusts and leaf blotch pathogens (Dutzmann, 1997).

1.5.2.4 Inhibitors of ergosterol 3-ketoreductase

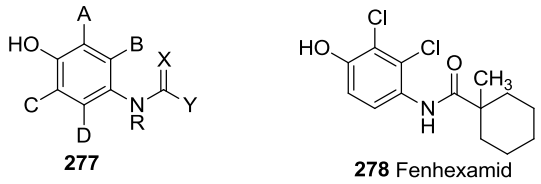
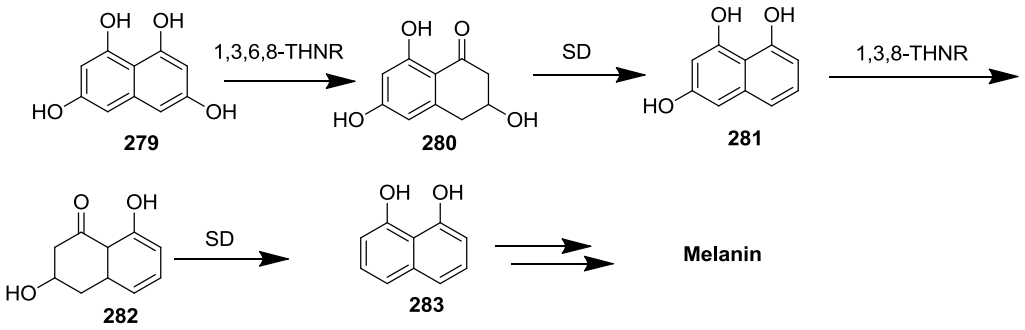


Figure 1.22 Inhibitors of ergosterol 3-ketoreductase **277** and **278** (Kruger, Etzel et al., 1999; Debieu, Bach et al., 2001).

In 1999, Bayer introduced a new fungicide fenhexamid (**278**) optimized from compounds with a general structure **277**. Compounds having the cycloalkyl substituent instead of the 'Y' group, a dihalosubstituted phenyl ring, and a free 'NH' group in the general structure **277** exhibited fungicidal activity against *B. cinerea* (Figure **1.22**) (Kruger, Etzel et al., 1999). Later Debieu, Bach et al. (2001) showed that fenhexamid (**278**) inhibits sterol biosynthesis by a totally different mode as compared to other known fungicides. It was reported that compound **278** inhibited 3-keto reductase involved in the C-4 demethylation of compound **247** in ergosterol biosynthesis (Scheme **1.21**) (Debieu, Bach et al., 2001).

1.5.3 Inhibitors of melanin biosynthesis



Scheme 1.22 Selected steps in fungal melanin biosynthetic pathway carried out with the help of 1,3,6,8-tetrahydroxy naphthalene reductase (1,3,6,8-THNR), 1,3,6,8-trihydroxy naphthalene reductase (1,3,8-THNR) and scytalone dehydratases (SD) (Butler and Day, 1998).

Melanin in mammals is biosynthesized from tyrosine and 3,4-dihydroxyphenylalanine (L-DOPA), whereas in fungi melanin derives from acetyl and malonyl CoA through polyketide biosynthesis (Butler and Day, 1998). 1,3,6,8-Tetrahydroxynaphthalene (**279**), scytalone (**280**), 1,3,8-trihydroxynaphthalene (**281**), vermelone (**282)** and 1,8-dihydroxynaphthalene (**283)** are important intermediates in the fungal biosynthesis of melanin (Scheme **1.22**). Most of the enzymes involved in the melanin biosynthesis pathway are well characterized (Butler and Day, 1998). Design of melanin biosynthesis inhibitors is mainly based on two target enzymes: the reductases 1,3,6,8-tetrahydroxynaphthalene reductase, 1,3,8-trihydroxynaphthalene reductase (phthalide, tricyclazole, pyroquilone) and scytalone dehydratase (carpropamid, dicyclocymet, fenoxanil). Besides the inhibitors of the above two enzymes, two natural products (Figure **1.23**) were identified as potent inhibitors of the polyketide synthase. The first one, aflastatin A (**284**), was a compound isolated from *Streptomyces* species (Ono, Sakuda et al., 1997). Studies of Okamoto et al. revealed that the compound **284** inhibits early steps in the melanin biosynthesis (Okamoto, Sakurada et al., 2001). The second compound abikoviromycin (**285**) was isolated from *Colletotrichum lagenarium* (Maruyama, Okamoto et al., 2003).

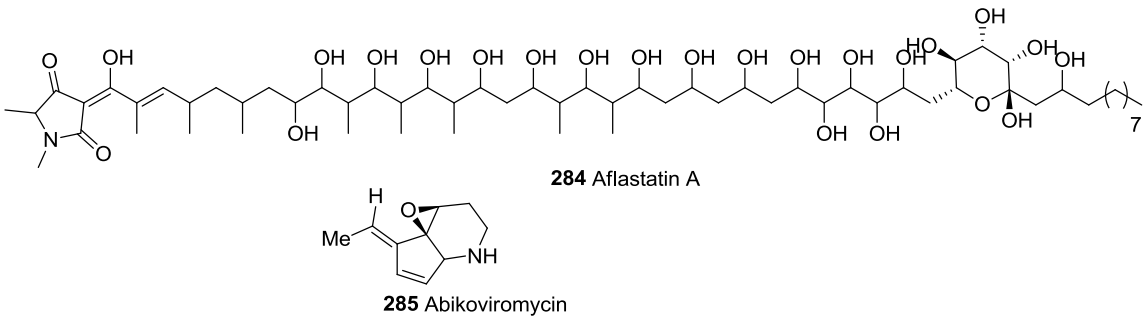


Figure 1.23 Inhibitors of melanin biosynthesis: natural products aflastatin A (**284**) (Okamoto, Sakurada et al., 2001) and abikoviromycin (**285**) (Maruyama, Okamoto et al., 2003).

1.5.3.1 Inhibitors of trihydroxynaphthalene reductases

Wheeler and Gleenblatt (1988) showed that some of the inhibitors of melanin biosynthesis in appressoria of *Pyricularia oryzae* were as well inhibitors of reductases involved in conversion of 1,3,6,8-tetrahydroxynaphthalene (**279**) and 1,3,8-trihydroxynaphthalene (**281**) to scytalone (**280**) and vermelone (**282**), respectively (Wheeler and Greenblatt, 1988). However, these compounds did not inhibit enzymatic reactions that dehydrate scytalone (**280**) and vermelone (**282**). This suggested that these compounds were inhibitors of only the reductases and not of dehydratases in the melanin biosynthetic pathway (Wheeler and Greenblatt, 1988). Among these compounds, three of the strongest inhibitors of trihydroxynaphthalene reductase activity, namely tricyclazole (**286**), phthalide (**287**) and pyroquillon (**288**), are used as commercial fungicides to treat rice blast disease (Figure 1.24) (Liao, Basarab et al., 2001). Binding of the inhibitors phthalide (**287**) and pyroquilone (**288**) to the active site was explained using X-ray crystal structures of the inhibitors bound to 1,3,6,8-tetrahydroxynaphthalene reductase (Liao, Basarab et al., 2001).

Jordan and co-workers used a computational program and developed a docking model to design and score several compounds as potential inhibitors of 1,3,8-trihydroxynaphthalene reductase. The shape of the active site, lipophilicity and

hydrogen bond donation were the important properties that were explored in the design process. Functional groups that append to the planar, lipophilic core were chosen (Jordan, Basarab et al., 2001). Based on these criteria several analogs were designed which exhibited a range of K_i values including nano molar potencies. A good correlation was observed in case of a few inhibitors between the predicted and observed inhibition of trihydroxynaphthalene reductase values. The most potent inhibitors of this class were compounds **290**-**294** (Figure **1.24**) (Jordan, Basarab et al., 2001).

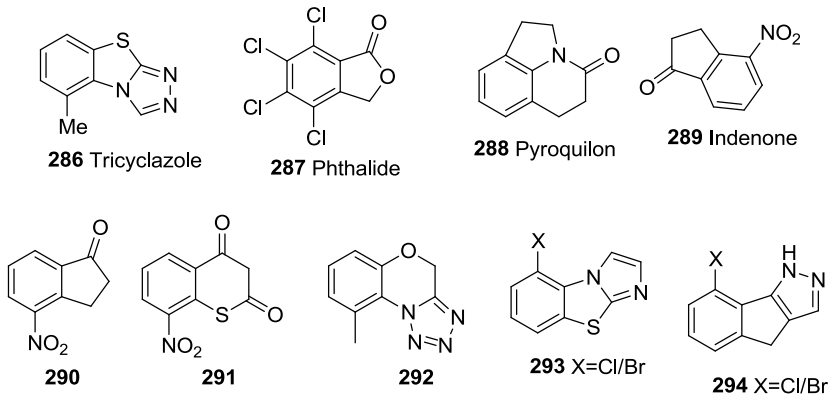


Figure 1.24 Inhibitors of trihydroxynaphthalene reductase **286**-**289** (Liao, Basarab et al., 2001) and **290**-**294** (Jordan, Basarab et al., 2001).

Recently, Brunskole and co-workers used a series of compounds and identified a few structurally diverse inhibitors **295**-**299** of trihydroxynaphthalene reductase (Figure **1.25**) from *Curvularia lunata*, a known plant pathogen. Inhibitor activities in these studies were explored by homology modeling of the active site of *C. lunata* (Brunskole, Stefane et al., 2008).

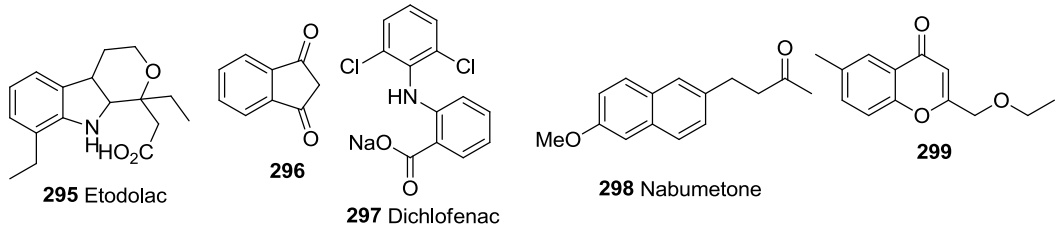


Figure 1.25 Inhibitors of trihydroxynaphthalene reductase **295**-**299** (Brunskole, Stefane et al., 2008).

1.5.3.2 Inhibitors of scytalone dehydratase

Scytalone dehydratases catalyze the conversion of scytalone (**280**) and vermalone (**282**) to compounds **281** and **283**, respectively, in the fungal melanin biosynthetic pathways (Scheme **1.22**). Scytalone dehydratase (SD) is an important agrochemical target from the *Magnaporthe grisea*, which causes blast disease of rice. It was found that *M. grisea* mutants devoid of scytalone dehydratase were unable to infect the rice plants (Chumley and Valent, 1990).

The first inhibitor of scytalone dehydratase, a salicylamide **300**, was designed based on the structure of substrate **280** and the probable enzymatic mechanism (Hodge and Pierce, 1993). Peryhydroxy carbonyl moiety involved in H-bonding was important for activity. SAR studies suggested that the compounds resulted from replacement of the hydroxyl group in compound **300** with F, Cl or OMe groups resulted in loss of activity by two folds (Hodge and Pierce, 1993). Replacement of a cyclic hydrogen bond array in compound **300** with a phenazine ring made compound **304** an equally potent (nanomolar) inhibitor of SD. Inhibitor design in this work was guided through the isosteric replacement of the active chemical ingredient such as the peryhydroxybenzoyl group with a quinazoline moiety (Hodge and Pierce, 1993). 4-aminoquinazoline as the bioisostere of the salicylamide moiety was exploited by Chen and co-workers, who tried several replacements of heterocyclics in structure **300** (Chen, Xu et al., 1998). Compounds **303a** and **304a** effectively inhibited SD among the compounds **302a**-**305a** that were obtained with heterocyclic replacement in compound **300** with quinolines, isoquinolines, quinazolines and benzotriazines (Figure **1.26**). Further, a more potent lead inhibitor of SD, **301** was used in optimization studies (Jennings, Wawrzak et al., 1999).

Replacement of the side chain in compound **301** by the 3,3-diphenylpropyl moiety resulted in an increase of activity in compounds **304c** and **305c**. Modification of structure **304c** by substitution of the nitrile group, as in compound **306**, further

improved the SD inhibitory activity (Chen, Xu et al., 1998). Chen, Xu et al. (1998) observed a similar effect with compound **307** that was optimized from triazine structure **305c** (Figure **1.26**). The increased activity was due to displacement of a water molecule in the active site with a nitrile group, which was evident from the crystal structure (Chen, Xu et al., 1998; Walter, 2002).

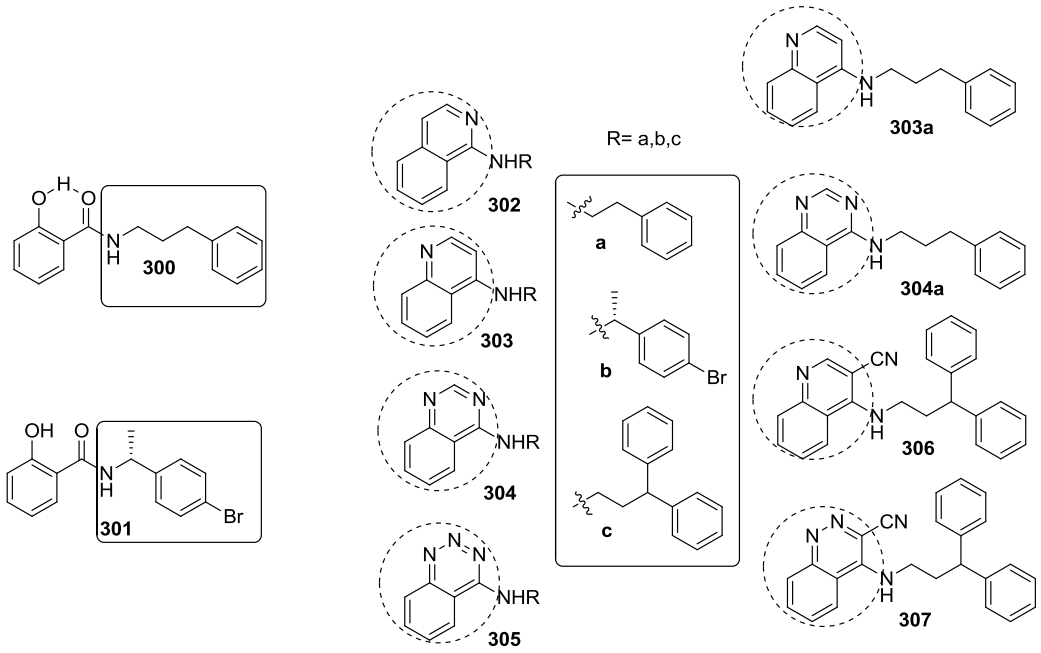


Figure 1.26 Design of inhibitors of scytalone dehydratase **302-307** based on the structure of compounds **300** and **301** (Chen, Xu et al., 1998; Hodge and Pierce, 1993; Walter, 2002).

Jennings and co-workers retained the amide functional group among their newly designed inhibitor compounds since the amide group was important in hydrogen bonding (Jennings, Wawrzak et al., 1999). Using the combinatorial chemistry of amines from the previous lead compounds and several acids, a picomolar potent inhibitor of SD **308** was identified (Jennings, Wawrzak et al., 1999). During these studies various amides, synthesized from coupling of (*R*)-α-methyl-4-bromobenzylamine and other amines with several acids, were initially screened as mixtures, and then more potent compounds were screened separately (Jennings, Wawrzak et al., 1999). Basarab noted that compound **309** was a potent inhibitor (47 pM) of SD but, that it was detoxified *by M. grisea* (Basarab, Jordan et al., 1999; Jordan,

Lessen et al., 1999). However, when the substitutuent on the amide moiety in **309** was replaced by a phenoxypropyl group, a stable and more potent inhibitor **310** was resulted. Further, two phenoxypropylsalicylamides **313** and **314** were designed based on the structures of known fungicides dicyclomet (**311**) and carpropamid (**312**) (Jordan, Lessen et al., 1999). Especially, compound **314**, a structural relative of dicyclomet (**311**), was a very effective inhibitor of SD. Further, based on the structure of compound **314**, and thorough analysis of binding interactions of potent inhibitors with SD, replacement of the t-butyl group by smaller groups and modification of the amide substituent by introducing additional phenyl substituents were considered (Figure **1.27**). This strategy resulted in the series of norephedrine analogs with more potent inhibitors such as **315** and **316** (Basarab, Jordan et al., 1999).

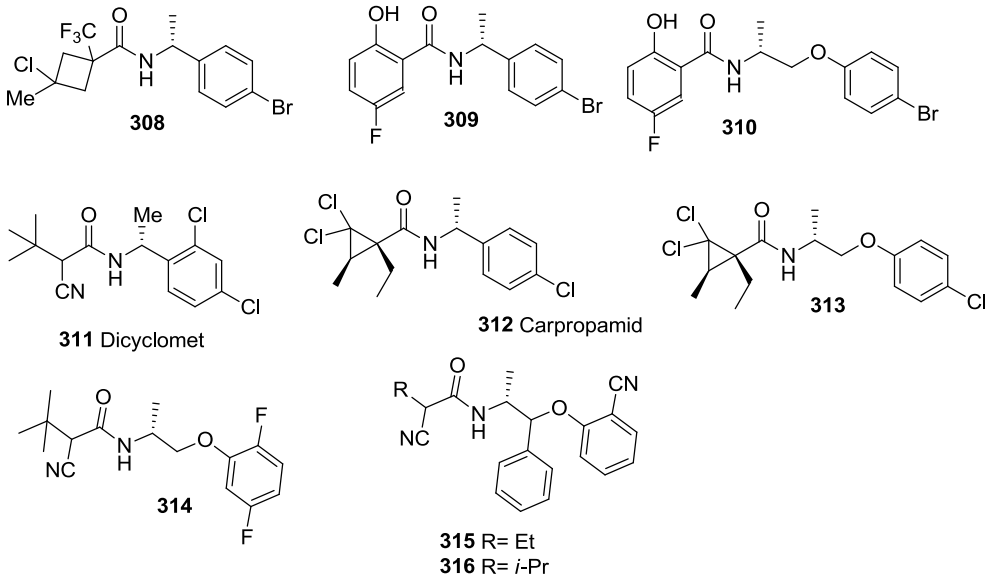


Figure 1.27 Inhibitors of scytalone dehydratase **308**-**316** (Basarab, Jordan et al., 1999; Jennings, Wawrzak et al., 1999; Jordan, Lessen et al., 1999).

1.5.4 Impact of fungicidal usage

Fungicides are benificial to improve the quality and yield of crops that are prone to fungal diseases. Thus, with the growing demand for food, production of fungicides is equally important. Kleffman reported that fungicidal usage on oilseed rape increased by

16% in the UK between 2009 and 2011 (Kelffmann, 2009). However, the negative impact of fungicide use has been recognized (Dewaard, Georgopoulos et al., 1993; Russell, 2005; Yang, Hamel et al., 2011). Fungicides such as ethylenebisdithiocarbamates **174**-**176** are known to be metabolized to ethylenethiourea, which is carcinogenic (Yang, Hamel et al., 2011). Residues of these fungicides have been found in several food samples and harvested crops, which is of great concern (Caldas, Conceicao et al., 2001). Excessive use of fungicides might lead to development of fungicide resistant fungal strains (Dewaard, Georgopoulos et al., 1993). Although there are fungicides against important targets, there is a continuous necessity for identification of new fungicides because of the pathogens' possible resistance mechanisms. Possible mechanisms of fungicide restance in fungi are: i) alteration of enzyme target sites; ii) synthesis of alternative enzymes to substitute the fungicide target enzyme; iii) overproduction of fungicide target; iv) reduced uptake of the fungicide; and v) metabolic breakdown (Ma and Michailides, 2005). For example, resistance to inhibitors of respiratory complexes is known among some pathogenic fungi (Leroux, Gredt et al., 2010). Similarly, carboxin derivatives acting as inhibitors of succinate dehydrogenase (complex II) were also metabolized with the help of hydroxylation of aromatic hydrogens and methyl and methylene hydrogens. Resistance against scytalone dehydratase inhibitors is also known (Source: www.frac.info). In addition the use of fungicides could have an effect on the non-target soil microorganisms that indirectly help the crop production (Yang, Hamel et al., 2011). European scientists showed that massive usage of farm fungicides may be linked with negative effects of human resistance to pathogens (Enserink, 2009).

The availability of "omics" technology will be of help in identifying novel fungicidal targets (Fernandez Acero, Carbu et al., 2011). It is possible that the chances of developing resistance to the inhibitors of primary metabolism in pathogens is higher when compared to inhibitors of fungal secondary metabolism such as detoxification of phytoalexins. In that way paldoxins may be more beneficial than conventional fungicides.

1.6 Conclusions

Chemistry and Biochemistry aspects involved in plant-pathogen interactions are very complex, and a thorough understanding of those would be helpful in implementing measures for controlling plant pathogens. Enzymatic detoxification of plant defense metabolites such as phytoalexins is an important strategy adopted among several crucifer pathogens. As a consequence, plants are deprived of their natural defenses and would depend on the unnatural defenses. Although, there are different modes of blackleg disease treatments, chemical and non-chemical both, available to treat *L. maculans*, these methods are not sustainable. Especially, despite the effectiveness of the fungicides in controlling fungal diseases, several pathogens acquire resistance to these chemicals, making them less viable. In this regard, paldoxins might serve as better crop protecting agents, provided the phytoalexin detoxification inhibitors are designed and optimized as per the desired properties including low antifungal and cytotoxic activities.

This thesis presents investigations of the Chemistry involved in metabolism of phytoalexins and related compounds by *L. maculans* (virulent on canola) and the effects of quinoline and isoquinoline derivatives on phytoalexin detoxification.

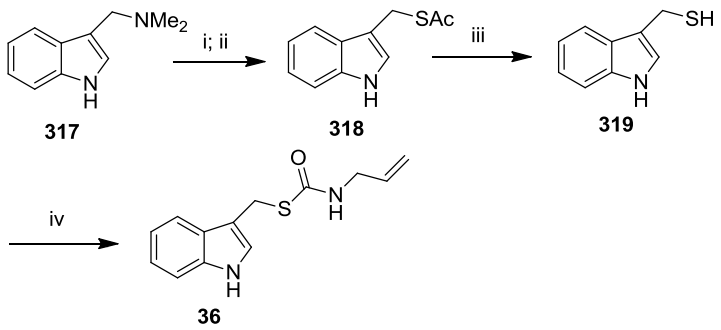
Chapter 2: Results and discussion

2.1 Phytoalexins and analogs

2.1.1 Synthesis

2.1.1.1 Phytoalexins

To study the biological activities of phytoalexins, it is essential to have these compounds available in sufficient quantities. Synthetic routes for all the published cruciferous phytoalexins have been established and the work has been reviewed (Pedras, Yaya et al., 2011c; Pedras, Zheng et al., 2007e). In my current work, phytoalexins were synthesized based on the best available published procedures. Syntheses of brassinin (**17**) (Kutschy, Dzurilla et al., 1998; Pedras, Borgmann et al., 1992), rapalexin A (**22**) (Pedras, Zheng et al., 2007d), and erucalexin (**29**) (Pedras, Suchy et al., 2006) were carried out as shown in experimental procedures according to the published procedures. Brussalexin (**36**) was synthesized by a modification of the previously reported synthetic procedure (Pedras, Zheng et al., 2007f). The modified route includes the synthesis of brussalexin (**36**) through the indolyl-3-methanethiol (**319**) intermediate as shown in the scheme **2.1**. First, thioacetate **318** was prepared starting from grammine (**317**) according to a published procedure (Benghait, Crooks, 1983). Next, the crucial step involved the hydrolysis of unprotected thioacetate **318** using hydrazine hydrate (Tomlison, Grey et al., 2002), whereas the previous procedure had involved the hydrolysis of *N*-Boc protected **318**. Thus, in the absence of protection and deprotection steps, the new procedure uses a shorter route to access compound **36**. Synthesis of compound **319** is reported here for the first time following a transformation similar to that reported for the –SAc group (Tomlinson, Grey et al., 2002). Compound **319** is a potential intermediate which gives access to several indolyl-3-methanethiol derivatives, including brussalexin (**36**).

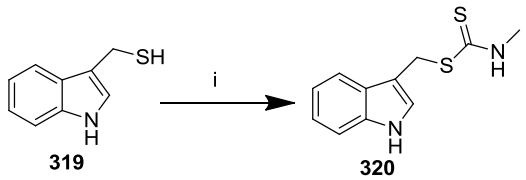


Scheme 2.1 Synthesis of brussalexin (**36**). Reagents and conditions: (i) Me_2SO_4, H_2O; (ii) CH_3COSH, KOH, 70 °C, 66% (Benghait and Crooks, 1983); (iii) $NH_2NH_2.H_2O$, THF, 80 °C, 84%; (iv) Et_3N, allyl isocyanate, DCM, 86%.

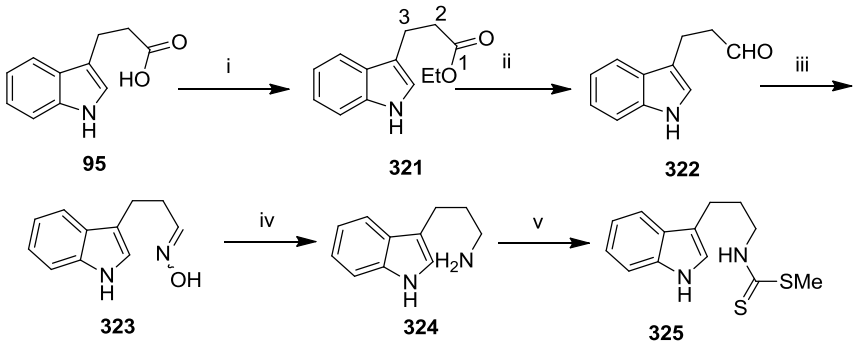
2.1.1.2 Phytoalexin analogs

Syntheses of the phytoalexin analogs, dithiocarbamates **96** (Pedras and Okanga, 2000), **111** and **342** (Pedras, Suchy et al., 2006), **325** (Gaspari, Banerjee et al., 2006), **327**, **331** and dithiocarbonates **153** (Pedras and Jha, 2006), **333** included common reaction conditions in the final step. They involved the treatment of the corresponding amines **155**, **324**, **326**, **330**, **339** and **343** or alcohols **156** and **332** with a base (Pyr, Et_3N or NaH) followed by reaction with CS_2 and MeI. The amines **324, 330**, **339** (Pedras, Suchy et al., 2006), **343** (Pedras, Suchy et al., 2006) and alcohol **332** were prepared according to the procedures described in the experimental section, whereas compounds **156** and **326** were purchased from commercial sources. Compounds **96** (Pedras and Okanga, 2000), **111** and **342** (Pedras, Suchy et al., 2006) were synthesized as previously reported. Methyl indolyl-3-methylcarbamodithioate (**320)** was synthesized starting from compound **319** in the presence of triethylamine and methyl isothiocyanate in 76% yield (Scheme **2.2**). Compound **325** was synthesized according to a general procedure used in the case of dithiocarbamate synthesis (Pedras, Yaya et al., 2011c). Oximes **323** were prepared starting from acid **95** in three steps according to a published procedure (Pedras, Minic et al., 2010b). Amine **324** was prepared by reduction of the oximes **323** using a $NiCl_2/NaBH_4$ system (Kutschy, Dzurilla et al., 1998), a procedure different from the reductive amination procedure used by Gaspari et al., in the synthesis of compound **324** (Gaspari, Banerjee et al., 2006). Finally, compound **325** was

prepared starting from amine **324** according to a general final step in the synthesis of dithiocarbamates, recently used by Gaspari et al. (Gaspari, Banerjee et al., 2006) (Scheme **2.3**).



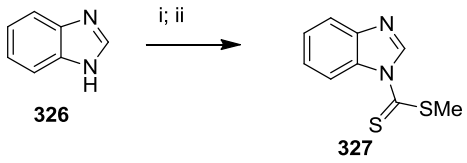
Scheme 2.2 Synthesis of methyl indolyl-3-methylcarbamodithioate (**320**). Reagents and conditions: (i) Et_3N, MeNCS, DCM, 76%.



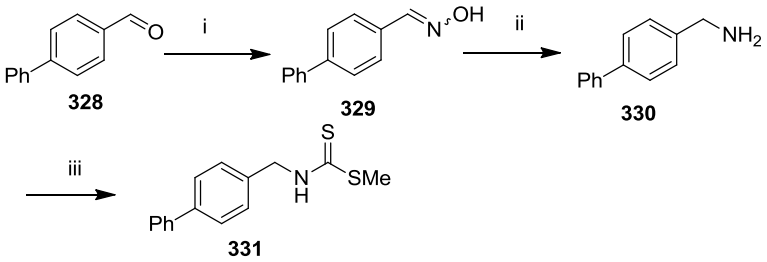
Scheme 2.3 Synthesis of methyl 3-(3-indolyl)propyldithiocarbamate (**325**). Reagents and conditions: (i) EtOH, H_2SO_4, 115°C, 96%; (ii) DiBAl-H, toluene, -78 °C, 90%; (iii) $NH_2OH.HCl$, Na_2CO_3, $EtOH/H_2O$, 97% (Pedras and Minic et al., 2010b); (iv) $NaBH_4$, $NiCl_2.6H_2O$, MeOH; (v) Pyr, Et_3N, CS_2, CH_3I, 43% (Gaspari, Banerjee et al., 2006).

In addition to homologues of brassinin (**17**), non-indolyl dithiocarbamates such as compounds **327** and **331** were synthesized using known methodology (Pedras, Jha et al., 2003b; Pedras and Jha, 2006; Pedras, Zheng et al., 2007e; Pedras, Yaya et al., 2011c). Methyl benzimidazolyldithiocarbamate (**327**) was synthesized starting from commercially available benzimidazole (**326**) in 72% yield (Scheme **2.4**). An alternative route for the synthesis of compound **327** was recently published, starting from compound **326** using NaOH as base, and a combination of CS_2 and dimethyl sulfate in DMSO in comparable yields (Devmurari, Shivanand et al., 2010). Methyl 4-biphenyldithiocarbamate (**331**) was synthesized using the same reagents as in the case of brassinin (Section **4.3.1.1**), starting from 4-biphenylcarboxaldehyde (**328**) instead of indolyl-3-carboxaldehyde (**74**) in 64% yield (Scheme **2.5**). It was found that yields of

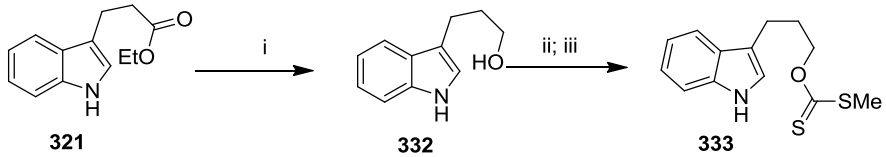
non-indolyl dithiocarbamates were higher than those of indolyl dithiocarbamates. Further, the analogs of brassinin **153** and **333,** with ethyl and propyl chains connecting the dithiocarbonate to 3-indolyl moieties were synthesized. Compound **153** was synthesized as previously reported in 90% yield (Pedras and Jha, 2006), whereas compound **333** was prepared starting from compound **321** in 69% yield (Scheme **2.6**). First, ester **321** (Pedras, Minic et al., 2010b) was reduced using $LiAlH_4$ to alcohol **332**, which was converted to dithiocarbonate **333** in the presence of NaH, CS_2 and MeI.



Scheme 2.4 Synthesis of methyl (benzimidazolyl)dithiocarbamate (**327**). Reagents and conditions: (i) NaH, THF, 0 °C; (ii) CS_2, CH_3I, 72%.



Scheme 2.5 Synthesis of methyl 4-biphenyldithiocarbamate (**331**). Reagents and conditions: (i) $NH_2OH.HCl$, Na_2CO_3, $EtOH/H_2O$, 97%; (ii) $NaBH_4$, $NiCl_2.6H_2O$, MeOH, 91%; (iii) Pyr, Et_3N, CS_2, CH_3I, 72%.



Scheme 2.6 Synthesis of methyl indolyl-3-propyldithiocarbonate (**333**). Reagents and conditions: (i) $LiAlH_4$, THF, 0 °C, 95%; (iii) NaH, THF, 0 °C; (iv) CS_2, CH_3I, 73%.

2.1.2 Antifungal activity

The choice of concentration of the compounds to be used in metabolism studies was made based on the antifungal activity of each compound. Phytoalexins and analogs were tested for antifungal activity against *L. maculans* at 0.50, 0.20 and 0.10 mM concentrations using a mycelial radial growth assay (Pedras and Jha, 2006). Solutions of each compound in ACN/DMSO (0.50 mM) were used to prepare assay solutions by serial dilution in PDA (potato dextrose agar), and control solutions were prepared with 1% ACN/DMSO in PDA. Further, the compound solutions and control solutions in PDA media were added to wells of 6-well sterile plates (35 mm well diameter, 2 ml/well). Mycelial plugs (4 mm) of *L. maculans* (virulent isolate UAMH 9410) grown on V_8 juice-agar media for 7 days were inoculated upside down in the centre of wells. After incubation of the plates under constant light for 7 days the growth of mycelia in each well was measured and used in the calculation of antifungal activities as described in the experimental section **4.5**. The antifungal activities of compounds **17**, **22**, **29**, **36**, **96**, **111**, **153**, **320**, **325**, **327**, **331**, **333** and **342** that were tested for metabolism in *L. maculans* are summarized in table **2.1**.

Brassinin (**17**) is considered a key compound among crucifer phytoalexins (Pedras, Yaya et al., 2011c). The structure of compound **17** contains a dithiocarbamate group linked to an indolyl moiety by a methylene unit. Some of the pesticides designed in 20th century were dithiocarbamate analogs. However, the antifungal activity of brassinin (**17**) cannot be attributed only to the dithiocarbamate functional group because methyldithiocarbamate itself does not possess antifungal activity against *L. maculans* (Pedras, Minic et al., 2011b). It is worth noting that the commercial fungicide mancozeb (**176**), which is ethylene bisdithiocarbamate, functions by a mode of action associated with multi-site activity (Gullino, Tinivella et al., 2010). The mode of action of compound **17** on crucifer pathogens remains to be established.

Table 2.1 Percentage of growth inhibition[a] of *Leptosphaeria maculans* incubated with phytoalexins **17**, **22**, **29**, **36** and analogs **96**, **111**, **153**, **320**, **325**, **327**, **331**, **333** and **342**.

Compound	Inhibition ± SD (%)[a]		
	0.50 mM	0.20 mM	0.10 mM
Brassinin (**17**)	55 ± 3	21 ± 4	10 ± 4
Rapalexin-A (**22**)	c.i.	53 ± 6	21 ± 6
Erucalexin (**29**)	40 ± 4	17 ± 4	6 ± 2
Brussalexin (**36**)	43 ± 3	25 ± 2	7 ± 4
Tryptaminedithiocarbamate (**96**)	c.i.	71 ± 4	43 ± 2
Isobrassinin (**111**)	44 ± 4	23 ± 4	13 ± 3
Tryptophol dithiocarbonate (**153)**	30 ± 3	18 ± 2	6 ± 3
Methyl indolyl-3-methylcarbamodithioate (**320**)	61 ± 5	35 ± 3	17 ± 3
Methyl 3-(3-indolyl)propyldithiocarbamate (**325**)	67 ± 3	46 ± 5	21 ± 5
Methyl benzimidazolyl dithiocarbamate (**327**)	c.i.	45 ± 4	32 ± 7
Methyl 4-biphenyl dithiocarbamate (**331**)	46 ± 4	32 ± 4	14 ± 4
Methyl 3-(3-indolyl)propyldithiocarbonate (**333**)	32 ± 4	15 ± 2	7 ± 3
1-Methoxyisobrassinin (**342**)	34 ± 3	15 ± 3	7 ± 3

[a] The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth on amended/growth in control) × 100]; c.i. = complete inhibition.

Results of antifungal bioassays showed that the phytoalexins erucalexin (**29**) and brussalexin (**36**) were less potent inhibiting the fungal growth than brassinin (**17**), while rapalexin A (**22**) displayed higher inhibition than brassinin. Among the brassinin homologues, both **96** and **325** were more inhibitory against the fungal growth than brassinin (**17**). Methyl tryptaminedithiocarbamate (**96**) showed complete inhibition of the fungal growth at 0.50 mM, while compound **325** showed about 70% growth inhibition at 0.50 mM. Dithiocarbamate **327** showed complete inhibition of fungal growth at 0.50 mM, whereas compounds **343**, **320, 111, 153, 331** and **333** were less potent than brassinin (**17**) at similar concentrations. The antifungal activities of compounds **17**, **22**, **29**, **36** and analogs **96**, **111**, **153**, **320**, **325**, **327**, **331**, **333** are summarized in table **2.1**.

2.1.3 Metabolism

2.1.3.1 Metabolism of phytoalexins

Rapalexin A (**22**) is a phytoalexin isolated from *B. rapa* (canola) upon elicitation with *A. candida* (Pedras, Zheng et al., 2007d). The antifungal bioassays determined that compound **22** was growth-inhibitory against *L. maculans* at 0.50 - 0.20 mM (isolates virulent on canola). Rapalexin A (**22**) was added to the fungal cultures of *L. maculans* and also to the uninoculated (control) medium at 0.10 mM. HPLC analysis of neutral, basic and acidic extracts revealed that compound **22** was extracted under neutral conditions and that no new metabolites were detected. Also, recovery of rapalexin A (**22**) from cultures and from the control samples was comparable (Figure **2.1**). These results indicated that rapalexin A (**22**) was not metabolized by *L. maculans*. So far, compound **22** is the third crucifer phytoalexin known to resist transformation by *L. maculans*, after spirobrassinin (**27**) (Pedras, Jha et al., 2005b) and camalexin (**32**) (Pedras, Jha et al., 2005b). Accumulation of these special phytoalexins in crucifer plants might offer additional protection against *L. maculans*. For this reason, it is important to further explore the defence metabolite profiles of crucifers infected with the blackleg fungus and identify the metabolites that resist transformation.

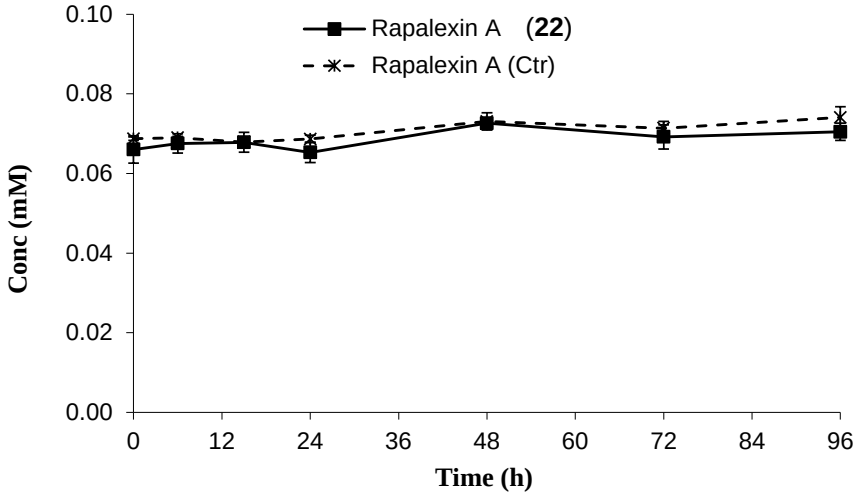


Figure 2.1 Curves representing the recovery of rapalexin A (**22**, 0.10 mM) in cultures of *Leptosphaeria maculans* in minimal medium and in control medium.

Erucalexin (**29**), a phytoalexin first isolated from *Erucastrum gallicum* (Pedras, Suchy et al., 2006), contains a spirocylic dithiocarbamate moiety attached to the indolyl derived unit at C-2. This compound **29** is a regio isomer of 1-methoxyspirobrassinin (**28**). Erucalexin (**29**) is known to be antifungal against *R. solani* and *S. sclerotiorum* (Pedras, Suchy et al., 2006), but compound **29** showed less antifungal activity against *L. maculans* than brassinin (**17**). To determine if erucalexin (**29**) was transformed by *L. maculans*, this compound was incubated with fungal cultures of *L. maculans* (BJ-125). HPLC analyses of the broth extracts of cultures incubated with erucalexin (**29**) collected at different time intervals indicated it to be completely metabolized within 48 h; two new peaks were detected at t_R = 10.0 and 8.5 min. The compound with t_R = 10.0 was identified as demethoxyerucalexin (**335**) and the compound with t_R = 8.5 min as dihydroerucalexin (**334**). Compound **335** appeared to be formed earlier than compound **334** and in larger amount in cultures of *L. maculans* in minimal media; however, in cultures of *L. maculans* in water, compound **334** was detected as the major metabolite and compound **335** was present in negligible quantities. Attempts to recover compound **334** from fungal cultures in minimal media resulted in its degradation to demethoxyerucalexin (**335**). Compound **334** could only be recovered from fungal cultures in water after freeze drying the broth. Finally, results from biotransformation experiments using mycelia of *L. maculans* in water incubated with erucalexin (**29**) demonstrated that compound **29** was metabolized to compound **334,** and that compound **335** resulted from its decomposition.

Confirmation of the structures of metabolites with t_R = 10.0 and 9.2 min was carried out using spectroscopic data. HPLC-ESI-MS analysis of the samples indicated that the metabolite at t_R = 10.0 min had a mass of 250.0 Da, which is 30 mass units less than erucalexin (**29**). ^{1}H NMR analysis of the broth extract indicated the presence of one methyl group and an intact spirocyclic system, as in compound **29**. Overall, the ^{1}H NMR spectral pattern of the compound with t_R = 10.0 min was similar to that of compound **29** except for the absence of the signal due to N-OMe. Thus, demethoxylated product of **29** seemed a probable structure. Upon comparison with a synthetic sample of demethoxyerucalexin (**335**), the unknown metabolite with t_R = 10.0 min was identified as compound **335**.

HPLC-ESI-MS analysis indicated that the metabolite with t_R = 9.2 min had a base peak corresponding to 250 Da and a weak fragment at 282 Da. Adequate quantities of this metabolite (t_R = 9.2 min) were not formed when fungal cultures in minimal media were incubated with compound **29**. Moreover, demethoxyerucalexin (**335**) was formed in larger amounts before the metabolite with t_R = 9.2 min was detected in the cultures. Overall, it seemed that erucalexin (**29**) was transformed to compound with t_R = 9.2 min via demethoxyerucalexin (**335**). However, no formation of demethoxyerucalexin (**335)** was detected when it was incubated in fungal cultures in minimal media. When compound **29** was incubated in fungal cultures in water, compound with t_R = 9.2 min was detected as the major metabolite, with negligible amounts of demethoxyerucalexin (**335**). ^{1}H NMR spectrum of freeze-dried extract of the 48 h samples indicated a complex mixture of sirodesmins and a distinct spirocyclic system similar to erucalexin (**29**). Attempts to purify the unknown metabolite by FCC on silica gel or alumina were unsuccessful, resulting in decomposition of the compound with t_R = 9.2 min to demethoxyerucalexin (**335**). Finally, compound with t_R = 9.2 min was purified by FCC using a reversed phase silica gel column.

The ^{1}H NMR spectrum of the purified metabolite with t_R = 9.2 min showed a –SMe group, a -OMe group and a spirocyclic system as in compound **29**. However, this compound showed, relative to the ^{1}H NMR spectrum of erucalexin (**29**), two additional peaks at 4.94 and 4.09 ppm, with the peak at 4.09 ppm due to an exchangeable proton. HRMS-EI analysis indicated that the unknown metabolite had a molecular formula $C_{12}H_{14}N_2O_2S_2$ which was two mass units higher than erucalexin (**29**). The probable structure of the unknown metabolite was identified as dihydroerucalexin (**334**) which was further confirmed by synthesis. Thus, compound **334** was identified as the product of erucalexin (**29**) reduction by *L. maculans*.

Synthesis of dihydroerucalexin (**334**) using $NaBH_4$ reduction of racemic erucalexin (**29**) resulted in the formation of compounds **334a** and **334b** as a mixture of diastereomers in 3:1 ratio. The major isomer could be isolated in pure form, while the minor isomer was purified only up to a 1:1 mixture of diastereomers. Further, it was important to determine the stereoselectivity of the reduction of erucalexin (**29**) in

fungal cultures. The ^{1}H NMR spectrum of freeze-dried crude extract of the fungal cultures incubated with erucalexin (**29**) indicated two sets of proton signals corresponding to the major and minor diastereomers in 2:1 ratio. It was observed that the same major product was formed in both chemical and enzymatic reductions of racemic erucalexin (**29**).

Further, the relative stereochemistry in each diastereomer **334a** and **334b** was established based on the results of nuclear Overhauser effect (NOE) experiments and 3-D modeling of compound **334** (Figure **2.2**). NOE difference experiments were carried out using a 3:1 mixture of diastereomers **334a** and **334b**. When the methine proton of the major diastereomer at 4.95 ppm was irradiated, the signal intensity (0.3%) of the methylene proton at 4.26 ppm was increased (Part C). Likewise, when the methylene proton of the major diastereomer at 4.26 ppm was irradiated, the signal intensity (0.05%) of the methine proton at 4.95 ppm was increased (Part D). On the other hand, when the methine proton at 5.24 ppm of the minor isomer was irradiated no NOE was observed on any of the methylene protons (Part B). Further, atomic distances between corresponding methine (H_a/H_b) and methylene protons (H_R/H_S) in the possible diastereomers (2*S*,3*S*)-3-dihydroerucalexin (**334a**) and (2*S*,3*R*)-3-dihydroerucalexin (**334b**) revealed that only in compound **334a** (and its enantiomer) the two hydrogen atoms, i.e. H_a and H_S are in proximity (< 3.5 Å) to experience the NOE (Figure **2.2**). Consequently, the relative stereochemistry in the major diastereomer was assigned to be as in compound (2*S*,3*S*)-3-dihydroerucalexin (**334a**) or its enantiomer, in which H_a shares a cis relationship with H_S. On the other hand, in the minor isomer (2*S*,3*R*)-3-dihydroerucalexin (**334b**) (or its enantiomer) the two hydrogens, i.e. H_b and H_S, shared a trans geometry. The reason for preferential formation of one of the diastereomers over the other is not clear.

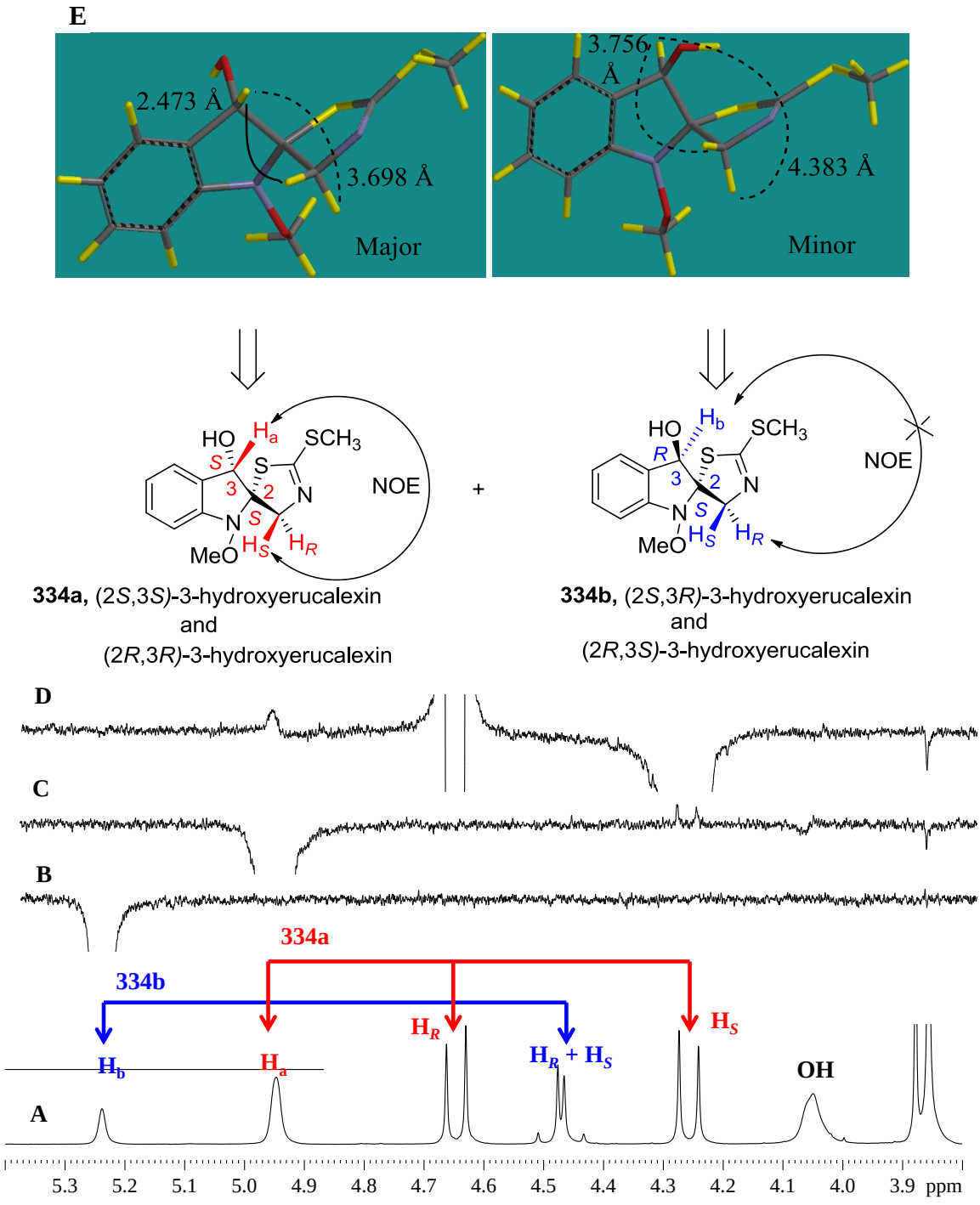


Figure 2.2 Relative stereochemistry in major and minor isomers of compound **334** determined based on 1-D NOE difference experiments and 3-D models of diastereomers (2*R*,3*R*)-3-dihydroerucalexin (**334a**) and (2*R*,3*S*)-3-dihydroerucalexin (**334b**). (A) Expansion of the ^{1}H NMR spectrum of **334a** and **334b**; (B) Irradiation of H_b of **334b**; (C) Irradiation of H_a of **334a**; (D) Irradiation of H_S of **334a**; (E) Analysis of NOE correlations based on the atomic distances in the models **334a** and **334b.**

Time-course metabolism studies revealed that erucalexin (**29**) was completely metabolized within 48 h while dihydroerucalexin (**334**) remained in the cultures (Figure **2.3**) and no further metabolite was detected (Scheme **2.7**). As described in section **1.4**, *L. maculans* (virulent on canola) is capable of producing several detoxifying enzymes, although there is no reported example of reduction of any cruciferous phytoalexins. Thus, reduction of erucalexin (**29**) is the first enzymatic transformation of its kind observed for *L. maculans* (virulent on canola).

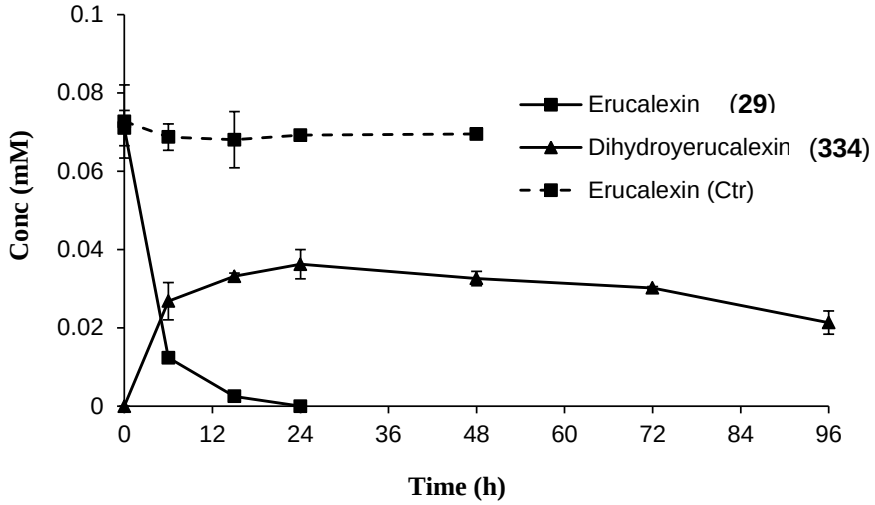


Figure 2.3 Progress curves of the transformation of erucalexin (**29**, 0.10 mM), formation of dihydroerucalexin (**334**) in mycelial cultures of *Leptosphaeria maculans* in water, and control erucalexin (**29)** in water.

Besides, it was not clear if in fungal cultures grown in minimal media, erucalexin (**29**) was transformed to compound **334** and demethoxyerucalexin (**335**) via two different pathways. The degradation of dihydroerucalexin (**334**) to demethoxyerucalexin (**335**) during its isolation from fungal cultures in minimal media suggested that compound **334** might be less stable in culture media. To further clarify this issue, control cultures of identical concentrations of **334** (0.20 mM) in minimal media and water were analyzed by HPLC. Analysis of the samples directly injected suggested that stability of compound **334** was similar in minimal media and in water. Next, control cultures of dihydroerucalexin (**334**) in minimal media and water at identical concentrations (0.20 mM) were extracted with EtOAc and the extract was analyzed by HPLC. Comparison of the HPLC chromatograms of the extracts indicated a lower recovery of compound

334 from minimal media than from water (Figure **2.4**). That is, the HPLC chromatograms of the extracts showed formation of higher amounts of demethoxyerucalexin (**335**) in minimal media than in water. This might suggest that components of the minimal media, e.g. metal ions, could catalyze the decomposition of dihydroerucalexin (**334**). Overall, these results demonstrated that demethoxyerucalexin (**335**) was not a product of erucalexin (**29**) metabolism by *L. maculans* (Scheme **2.7**).

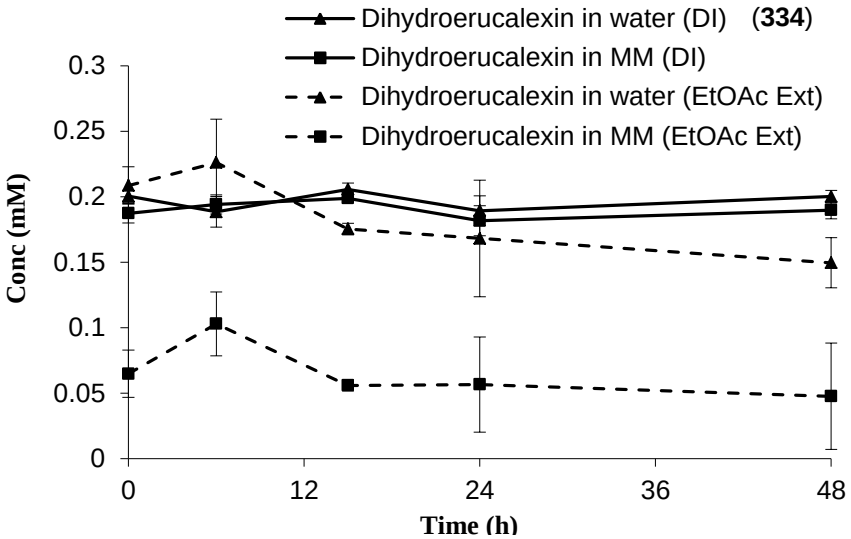
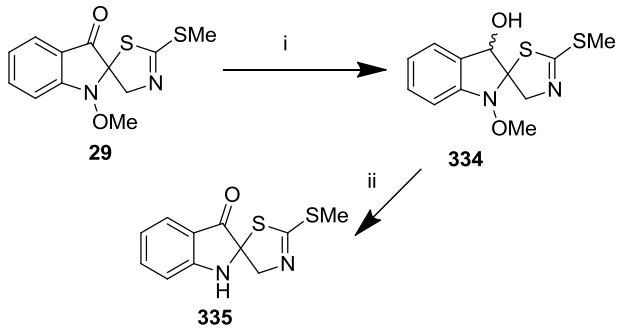
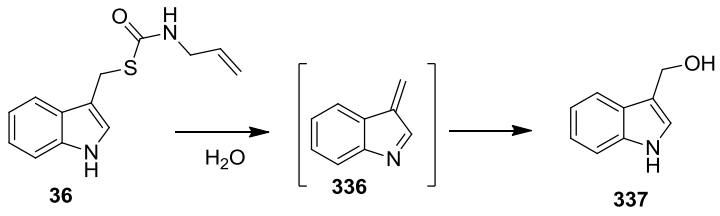


Figure 2.4 Progress curves of the recovery of dihydroerucalexin (**334**, 0.20 mM) in water and minimal media (MM). HPLC analysis was carried out by injecting the solutions of compound **334** in water and MM (DI: Direct Injection) or their ethylacetate extracts (EtOAc ext).



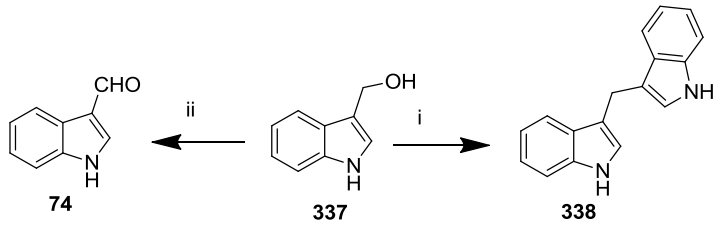
Scheme 2.7 (i) Biotransformation of erucalexin (**29**) by *Leptosphaeria maculans* and (ii) its non-enzymatic conversion.

Brussalexin (**36**), another phytoalexin isolated recently from Brussel sprouts, is known to be antifungal against several crucifer pathogens (Pedras, Zheng et al., 2007f) including *L. maculans*. So far, compound **36** is the only cruciferous phytoalexin derived from 3-indolylmethanethiol (**319**). Culture medium incubated with **36** revealed that the compound was not stable and was degraded to undetermined products. Further, stability studies performed in water determined that brussalexin (**36**) upon standing in aqueous solution decomposed to 3-indolylmethanol (**337**) via likely intermediate **336**, over a period of 72 h ($t_{1/2}$ = 24 h) (Scheme **2.8**). Although, under in vitro culture conditions compound **36** is not stable, **36** is stable in the infected plant which is evidenced by its isolation in reasonable amounts (Pedras, Zheng et al., 2007f). On the contrary, if part of the induced brussalexin (**36**) is degraded to compound **337**, further conversion of compound **337** to ascorbigen (**45**) and indole oligomers is expected (Agerbirk, Olsen et al., 1998). In this regard, compound **337** was tested for stability in water and MM. It was noted that compound **337** was not stable in MM and degraded to 3,3′-diindolylmethane (<6 h) (**338**), while transformed in water in very low quantities ($t_{1/2}$>72 h). Hence, the compound **337** was incubated with mycelia of *L. maculans* in water and it was observed that indole-3-carboxaldehyde (**74**) was also detected besides 3,3′-diindolylmethane (**338**), although in trace quantities (<2%). This suggested that aldehyde **74** was the enzymatic product of the metabolism of compound **337** by *L. maculans* (Scheme **2.9**).



36 **336** **337**

Scheme 2.8 Transformation of brussalexin (**36**) to 3-indolylmethanol (**337**).

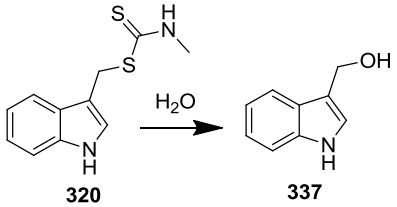


Scheme 2.9 (i) Decomposition of compound **337**; (ii) metabolism (traces) of compound **337** by *Leptosphaeria maculans*.

2.1.3.2 Metabolism of phytoalexin analogs

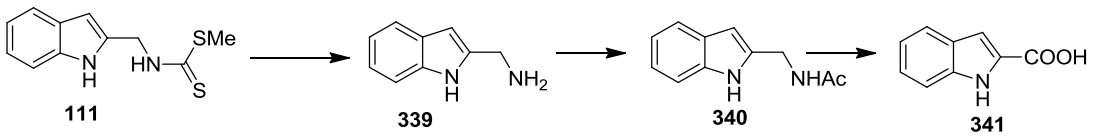
It is important to explore the compounds that resist metabolism by *L. maculans.* From the examples summarized in section **1.4.2**, it is clear that *L. maculans* can carry out a wide range of transformations. In continuation of previous work (Pedras and Okanga, 2000), dithiocarbamates **96**, **111**, **320**, **325**, **327**, **331**, **342** and dithiocarbonates **153, 333** bearing 2-indolyl, 3-indolyl, 4-biphenyl, and benzimidazolyl related moieties were investigated to determine their metabolism by *L. maculans* (BJ-125).

Firstly, the brassinin analog methyl indolyl-3-methylcarbamodithioate (**320**) having a dithiocarbamate group attached to the 3-indolyl moiety was used in the study. This compound, being a derivative of 3-indolylmethanethiol (**319**), is also an analog of brussalexin (**36**). More importantly, compound **320** shares a structural resemblance with brassinin (**17**). Compound **320** was not stable in aqueous media and degraded to 3-indolylmethanol (**337**) ($t_{1/2}$ = 20 h) (Scheme **2.10)**.

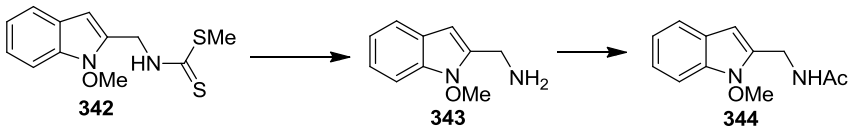


Scheme 2.10 Degradation of compound **320** to 3-indolylmethanol (**337**).

Next, metabolism of isobrassinin (**111**) in cultures of *L. maculans* (BJ-125) was investigated. Isobrassinin (**111**) is a regioisomer of brassinin (**17**) with an attachment of the dithiocarbamate side chain to a 2-indolylmethyl moiety. Isobrassinin (**111**, 0.10 mM) was completely metabolized within 48 h after incubation (Figure 2.5) in cultures of *L. maculans* (BJ-125). HPLC chromatograms of extracts of samples collected at 24 h indicated the presence of a metabolite (t_R= 4.6 min) that was transformed to another compound (t_R = 2.2 min) in 48-72 h. To establish the structures of these metabolites, larger scale cultures of *L. maculans* (isolate BJ-125) incubated with compound **111** were extracted and the extract was purified by FCC, followed by preparative thin layer chromatography (PTLC). Further, metabolites were identified and the pathway of biotransformation was established (Scheme **2.11** and scheme **2.12**).



Scheme 2.11 Biotransformation of isobrassinin (**111**) by *Leptosphaeria maculans*.



Scheme 2.12 Biotransformation of 1-methoxyisobrassinin (**342**) by *Leptosphaeria maculans*.

The structure of the metabolite with t_R = 4.5 min was determined by analysis of its spectroscopic data. The HRMS-EI spectral data indicated the molecular formula $C_{10}H_8N_2O_2$. ^{1}H NMR spectrum indicated the intact 2-substituted indole moiety accounting for the structure of N_b-acetyl-2-indolylmethanamine (**340**). Finally, the structure of the metabolite (t_R = 4.5 min) was confirmed by comparing with a synthetic sample. The metabolite with t_R = 2.2 min was identified as indole-2-carboxylic acid (**341**) based on ^{1}H NMR spectroscopy and HPLC-DAD comparison with an authentic sample. The potential intermediate and the first product of the metabolism of

isobrassinin (**111**) in cultures of *L. maculans* was proposed to be 2-indolylmethanamine (**339**), However, since compound **339** was not detected in the neutral extracts from cultures of *L. maculans* incubated with isobrassinin (**111**), samples were basified using 33% NH_4OH (pH~11) and extracted in 5% DCM/MeOH. The HPLC analysis of the basic extract indicated a peak at t_R = 3.1 min that was confirmed to be indole-2-methanamine (**339**) by comparison with a synthetic sample (Pedras, Suchy et al., 2006). HPLC analysis for the transformation of compound **111** to **339** and **340** showed that the amine **339** was not detected after 48 h and that the acetyl amine **340** was not detected after 96 h. Compound **111** was completely metabolized in 48 h (Figure **2.5**).

Further, to establish the pathway of transformation of isobrassinin (**111**), 2-indolylmethanamine (**339**) and N_b-acetyl-2-indolylmethanamine (**340**) were separately fed to cultures of *L. maculans* (BJ-125). HPLC chromatograms indicated that amine **339** was converted to acetamide **340** in 15-24 h. Compound **340** was completely converted to indole-2-carboxylic acid (**341**) after 96 h. Results of the metabolism of isobrassinin (**111**) indicated that it was metabolized at slower rate than brassinin (48 h vs 18 h). It is interesting to note that in cultures of *L. maculans* (BJ-125), compound **17** is metabolized to compound **74** through oxidation while its regioisomer **111** is transformed to amine **339**, through hydrolysis. It was shown that brassinin (**17**) was transformed to 3-indolylmethanamine (**76**) by a similar transformation in isolates of *L. maculans* (virulent on mustard). The enzyme involved, brassinin hydrolase (BH), has been isolated and characterized (Pedras, Minic et al., 2009b). The antifungal activities of the products formed during the metabolism of compound **111** by *L. maculans* (virulent on canola) are described in the following section.

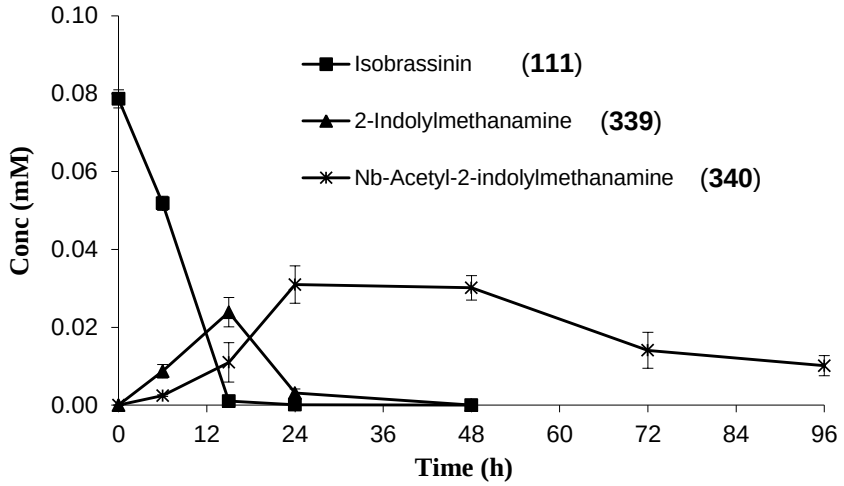


Figure 2.5 Progress curves of the metabolism of isobrassinin (**111**, 0.10 mM); formation of 2-indolylmethanamine (**339**) and N_b-acetyl-2-indolylmethanamine **(340)** in cultures of *Leptosphaeria maculans* grown in minimal media.

Next, 1-methoxyisobrassinin (**342**) was incubated in cultures of *L. maculans* (BJ-125). HPLC chromatograms of the neutral and basic extracts of the fungal cultures incubated with compound **342** were analyzed up to 72 h. HPLC-ESI-MS chromatograms of the extract of neutral samples revealed two peaks with t_R = 21.5 min (compound **342**) and t_R = 8.9 min. The molecular mass of the metabolite with t_R = 8.9 min was 218 Da, which suggested the structure of 1-methoxy-N_b-acetyl-2-indolylmethanamine (**344**). HPLC analysis of the basic extracts of the samples indicated the presence of 1-methoxy-2-indolylmethanamine (**343**). It was also observed that acetamide **344** accumulated in fungal cultures incubated with 1-methoxyisobrassinin (**342**) and no new metabolite was observed. To clarify if compound **344** is the final biotransformation product of **342** in *L. maculans*, it was incubated with fungal cultures and HPLC analysis of samples up to 72 h was conducted. These experiments revealed that compound **344** was not transformed. Thus, the pathway of biotransformation of compound **342** resembles that of compound **111** except for the final step (Scheme **2.11** and scheme **2.12**). The progress curves representing the metabolism of compound **342** and its metabolites are depicted in figure **2.6**. These transformations support the earlier observations that *L. maculans* does not transform aromatic rings.

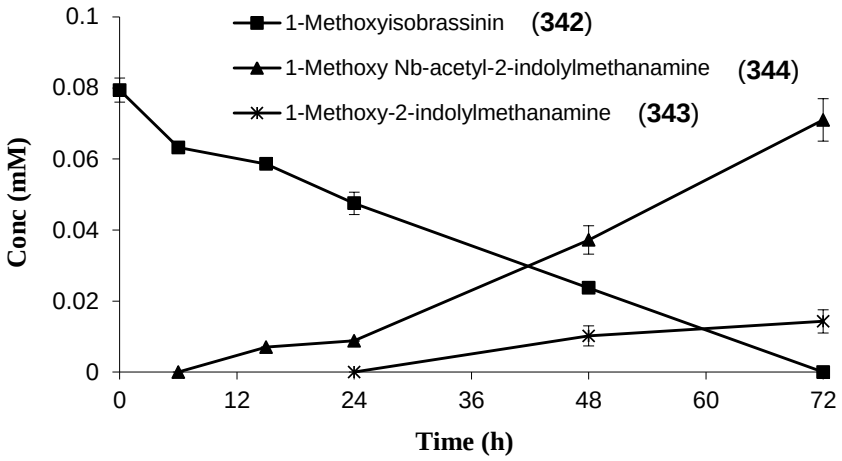
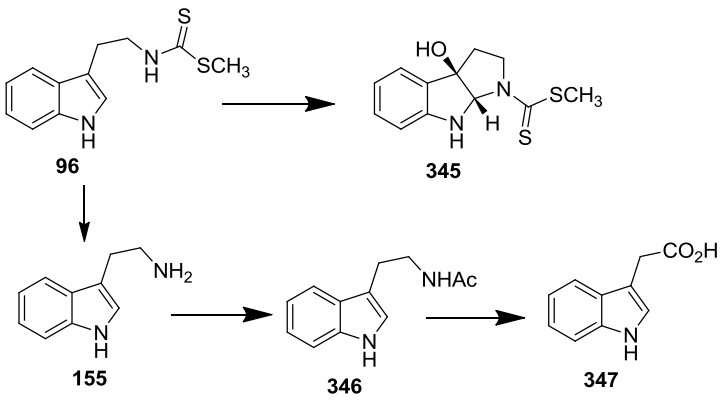


Figure 2.6 Progress curves of the metabolism of 1-methoxyisobrassinin (**342**, 0.10 mM); formation of 1-methoxy-2-indolylmethanamine (**343**) and 1-methoxy-N_b-acetyl-2-indolylmethanamine (**344**) in cultures of *Leptosphaeria maculans* grown in minimal media.

Previous studies (Pedras and Okanga, 2000) had shown that methyl tryptaminedithiocarbamate (**96**) was metabolized in cultures of *L. maculans* in a pathway different from that of brassinin (**17**). The sequence of steps involved in the detoxification of compound **96** was established; however, a time-course study of the metabolism of the compound **96** had not been published. Since such studies are important for comparison of the rates of metabolism among different phytoalexin analogs, compound **96** was incubated with cultures of *L. maculans* (BJ-125). Neutral, acidic and basic extracts of the samples collected at different time intervals were analyzed by HPLC and the metabolites **155**, **345-347** were detected and quantified (Figure **2.7**). The Results confirmed the previous observations that methyl tryptaminedithiocarbamate (**96**) is metabolized via two different pathways (Scheme **2.13**). Compound **345** was found to be a major product of metabolism, while compound **346** was also detected in reasonable amounts in cultures of *L. maculans* (BJ-125) incubated with compound **96.**



Scheme 2.13 Biotransformation of methyl tryptaminedithiocarbamate (**96**) by *Leptosphaeria maculans* (Pedras and Okanga, 2000).

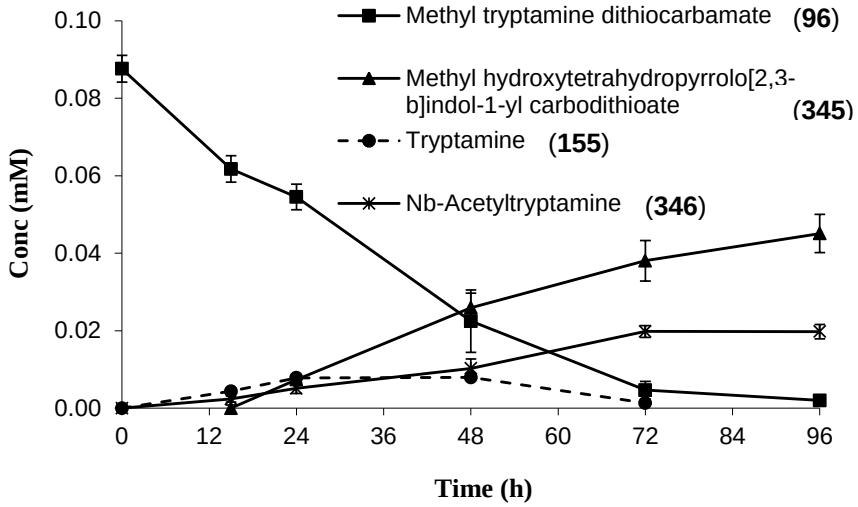
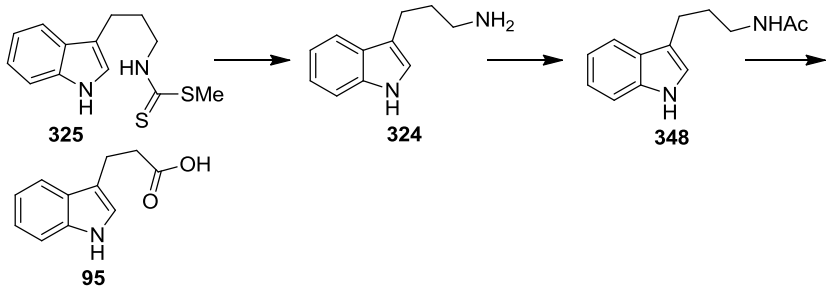


Figure 2.7 Progress curves of the metabolism of methyl tryptaminedithiocarbamate (**96**, 0.10 mM); formation of tryptamine (**155**), methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (**345**) and N_b-acetyltryptamine (**346**) in cultures of *Leptosphaeria maculans* grown in minimal media.

Next, methyl 3-(3-indolyl)propyldithiocarbamate (**325**) was added to cultures of *L. maculans*. Compound **325** is a homologue of brassinin (**17**) with two additional methylene units. Analysis of the HPLC and ESI-MS chromatograms of the neutral, acidic and basic extracts of the samples indicated that the products of transformation are compounds **95**, **324** and **348**. Unlike the homologue of brassinin, i.e. compound **96**, there was only one pathway detected in fungal cultures incubated with compound **325** (Scheme **2.14**). Compound **325** was completely metabolized in 72 h (Figure **2.8**).

Comparison of the metabolism rates of homologues of brassinin **96** and **325** in cultures of *L. maculans* (BJ-125) indicated that compound **96** was metabolized more slowly than compound **325**. It is likely that **96**, being highly antifungal against *L. maculans*, this compound induces multiple enzymes for faster detoxification of the same compound. This allows the fungus to be in contact with the antifungal compound for a minimum time.



Scheme 2.14 Biotransformation pathway of methyl 3-(3-indolyl)propyldithiocarbamate (**325**) by *Leptosphaeria maculans*.

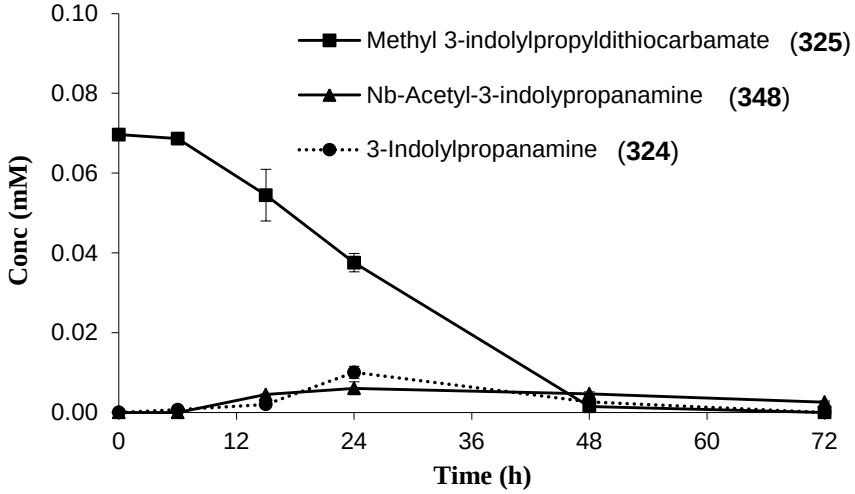
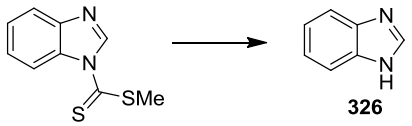


Figure 2.8 Progress curves of the metabolism of methyl 3-(3-indolyl)propyldithiocarbamate (**325**, 0.10 mM); formation of 3-indolylpropanamine (**324**) and N_b-acetyl-3-indolylpropanamine (**348**) in cultures of *Leptosphaeria maculans* grown in minimal media.

In addition to the indolyl analogs, dithiocarbamates bearing non-indolyl moieties were also analyzed for metabolism in *L. maculans*. Compound **327** has the dithiocarbamate functional group as part of the benzimidazole ring system. The compound showed very high antifungal activity against *L. maculans* (BJ-125) at the concentrations (0.50, 0.20, 0.10 mM) tested, and is more antifungal than brassinin (**17**). Compound **327** was incubated with cultures of *L. maculans* and samples were withdrawn up to 96 h. HPLC chromatograms of EtOAc extract of each sample indicated a new metabolite at t_R 4.2 min. Comparison with an authentic sample confirmed that the metabolite was benzimidazole (**326**), which was not metabolized (Scheme **2.15**) [confirmed by analysis of fungal cultures incubated with benzimidazole (**326**)]. In contrast to the primary amines **155**, **324**, **339** and **343** which are first products of dithiocarbamate metabolism, benzimidazole (**326**) is a secondary amine part of the aromatic system. It is likely that the fungal enzyme responsible for acetylation is ineffective in transforming compound **326**. Compound **327** was not detected in cultures of *L. maculans* (BJ-125) 72 h after the incubation, while **326** accumulated in the cultures up to 72 h (Figure **2.9**). These results suggest that enzymatic acetylation of amines takes place only in substrates fulfilling specific structural requirements.



327 **326**

Scheme 2.15 Biotransformation pathway of methyl benzimidazolyldithiocarbamate (**327**) by *Leptosphaeria maculans* in minimal media.

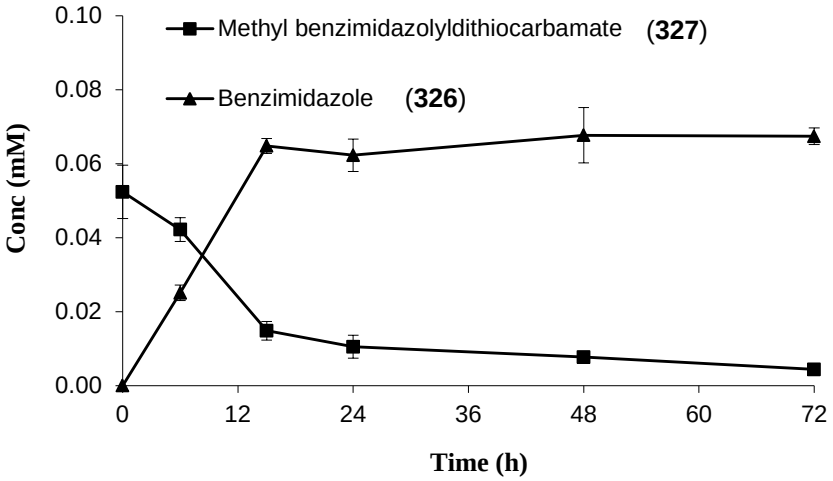
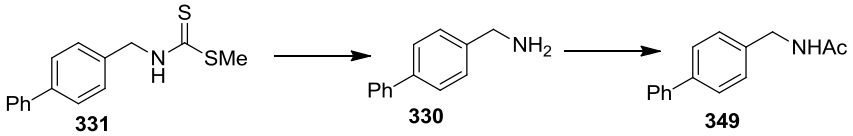


Figure 2.9 Progress curves of the metabolism of methyl benzimidazolyldithiocarbamate (**327**, 0.10 mM) and formation of benzimidazole (**326**) in cultures of *Leptosphaeria maculans* grown in minimal media**.**



Scheme 2.16 Biotransformation pathway of methyl 4-biphenyldithiocarbamate (**331**) by *Leptosphaeria maculans*.

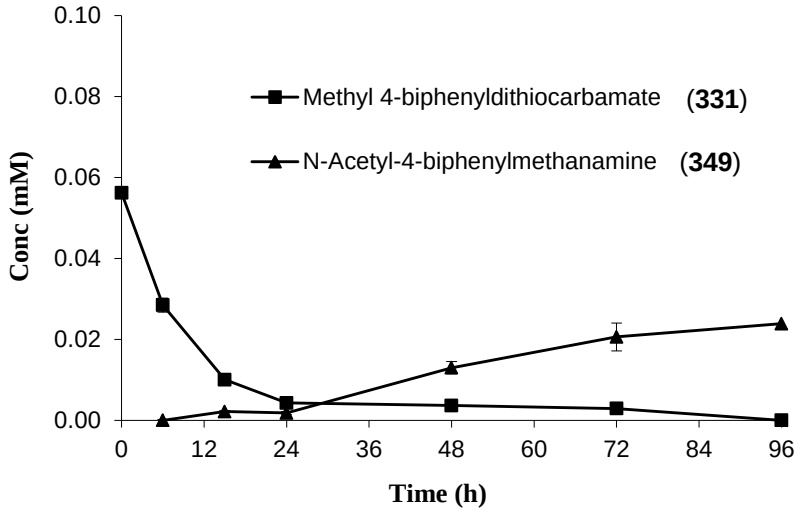
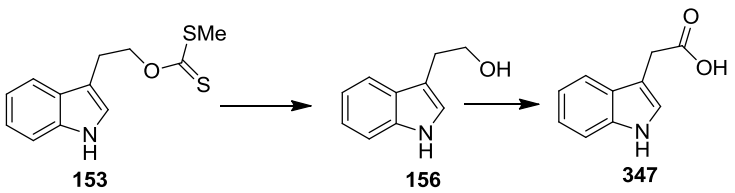


Figure 2.10 Progress curves of the metabolism of methyl 4-biphenyldithiocarbamate (**331**, 0.10 mM) and formation of *N*-acetyl-4-biphenylmethanamine (**349**) in cultures of *Leptosphaeria maculans* grown in minimal media.

Finally, methyl 4-biphenyldithiocarbamate (**331**) was incubated with cultures of *L. maculans* (BJ-125) and samples were withdrawn at different time intervals and analyzed by HPLC. HPLC chromatograms of the neutral, acidic and basic extracts of samples indicated a major metabolite with t_R = 18.3 min. HPLC-ESI-MS analysis of the metabolite followed by comparison with an authentic sample obtained by synthesis indicated that the metabolite was *N*-acetylbiphenylmethanamine (**349**). However, the first product of metabolism of 4-biphenylmethanamine (**330**) was not detected, probably due to its immediate conversion to compound **349**. Moreover, *N*-acetylbiphenylmethanamine (**349**) was not metabolized to any other product. The pathway of metabolism of compound **331** in *L. maculans* is shown in scheme **2.16**. Compound **331** was rapidly metabolized within 48 h, although it was detected in cultures in very low quantities up to 96 h. Accumulation of compound **349** was observed after 24 h period (Figure **2.10**). Results of the metabolism of indolyl and nonindolyl dithiocarbamates **111**, **320**, **325**, **327**, **331** and **342** suggested that the crucial first step in their detoxification process involves a hydrolysis reaction catalyzed by a hydrolase(s).

Next, indolyldithiocarbonates **153** and **333** were tested. Previous studies had suggested that methyl tryptopholdithiocarbonate (**153**) was metabolized to tryptophol (**156**) in cultures of *L. maculans* (Pedras and Jha, 2006). To further compare this transformation with compound **17**, compound **153** was incubated with cultures of *L. maculans* and EtOAc extracts of the samples collected at different time periods were analyzed by HPLC. HPLC chromatograms indicated that compound **153** was not detected after 48 h and tryptophol (**156**) accumulated in the cultures up to 96 h (Figure **2.11**). To verify if compound **156** is the final product in the pathway of tryptopholdithiocarbonate (**153**) metabolism, tryptophol was incubated with the cultures of *L. maculans* (BJ-125). Time course studies indicated that tryptophol (**156**) is further converted to indole-3-acetic acid (**347**), but at a very slow rate (Scheme **2.17**). More than 50% of tryptophol (**156**) was detected in cultures of *L. maculans* at 72 h after incubation.



Scheme 2.17 Biotransformation of methyl tryptopholdithiocarbonate (**153**) by *Leptosphaeria maculans*.

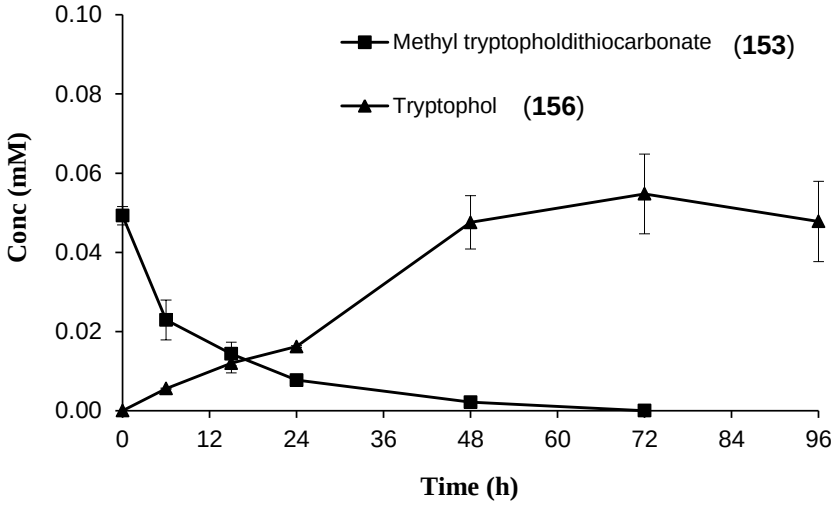


Figure 2.11 Progress curves of the metabolism of tryptophol dithiocarbonate (**153**, 0.05 mM) and formation of tryptophol (**156**) in cultures of *Leptosphaeria maculans* grown in minimal media.

Next, indolyldithiocarbonate **333** was incubated with cultures of *L. maculans*. Compound **333** is a homologue of compound **153** with one additional methylene unit. HPLC chromatograms of the neutral extract samples up to 72 h indicated the presence of a new metabolite with t_R = 7.2 min. Comparison of this metabolite with a standard sample indicated that it was 3-(3-indolyl)-1-propanol (**332**), which was metabolized to indole-3-propanoic acid (**95**). The sequence of steps involved in the metabolism of compound **333** by *L. maculans* is shown in scheme **2.18**. However, compound **333** was metabolized faster in cultures of *L. maculans* than compound **153** (24 h vs 72 h, figure **2.11** and figure **2.12**)**.** Comparison of the detoxification pathways of the compounds **153** and **333** (Scheme **2.17** and scheme **2.18**) showed that hydrolysis is the key reaction in the metabolism of indolyldithiocarbonates.

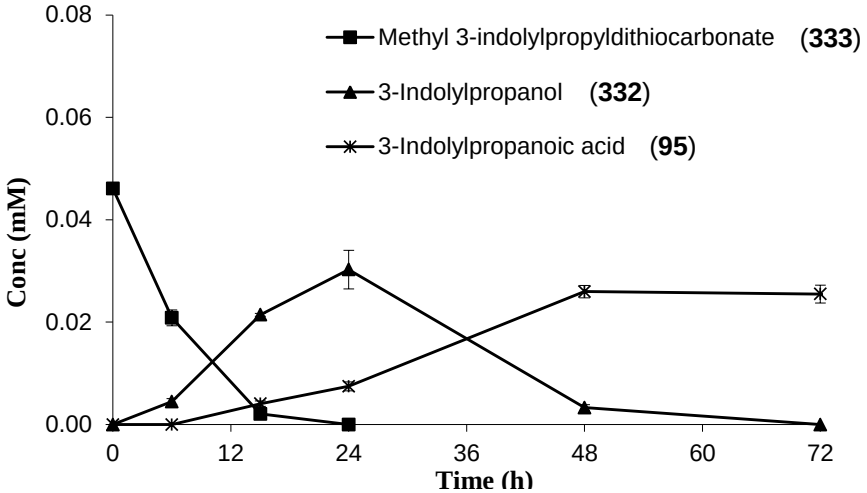
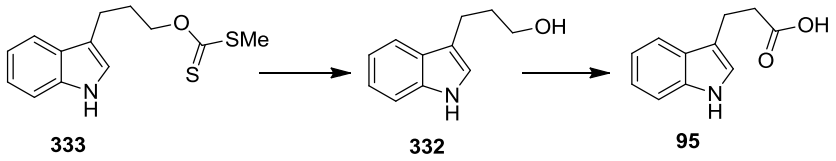


Figure 2.12 Progress curves of the metabolism of methyl 3-(3-indolyl)propyldithiocarbonate (**333**, 0.05 mM); formation of 3-(3-indolyl)propanoic acid (**95**) and 3-(3-indolyl)-1-propanol (**332**) in cultures of *Leptosphaeria maculans* grown in minimal media.



333 **332** **95**

Scheme 2.18 Biotransformation of methyl 3-(3-indolyl)propyldithiocarbonate (**334**) by *Leptosphaeria maculans*.

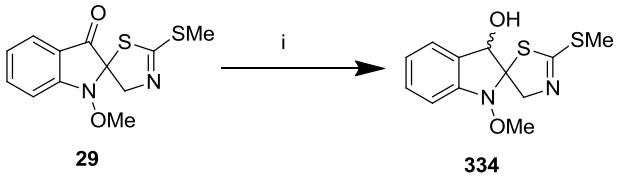
2.1.4 Synthesis and antifungal activity of metabolites

2.1.4.1 Synthesis

Dihydroerucalexin (**334**) is a product of erucalexin (**29**) metabolism in *L. maculans*. The structure of the metabolite was confirmed after comparison of the analytical data with a synthetic sample. Compound **334** was synthesized by reduction of erucalexin (**29**) in presence of $NaBH_4$ in methanol in 60% yield (Scheme **2.19**) as a 3:1 mixture of diastereomers. Compound **334** was purified on reversed phase column (C18), as it was unstable on silica gel and neutral alumina.

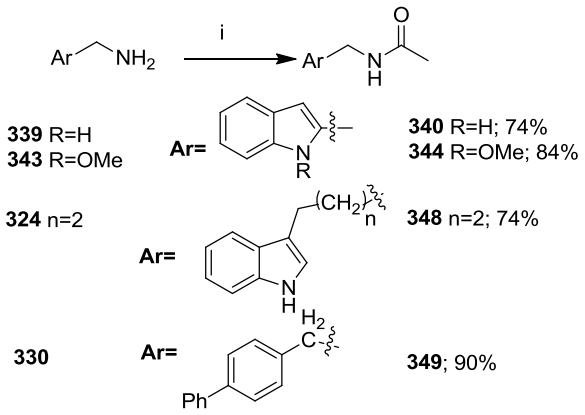
Synthesis of the tricyclic compound **345** was carried out as previously reported (Pedras and Okanga, 2000). 3-(3-Indolyl)propanol (**332**) was synthesized starting from indolyl-3-propanoic acid (**95**) as shown in the scheme **2.10**. Amines **324**, **330**, **339** and **343** are

synthetic precursors to dithiocarbamates and their preparation procedures are as described in the experimental section. N_b-Acetyltryptamine (**346**) was synthesized as previously reported (Pedras and Okanga, 2000) in 91% yield. Other acetylamines **340**, **344**, **348** and **349** were prepared according to the general procedure (Scheme **2.20**) by simple acetylation of amines using Pyr / Ac_2O in 74-90% yields.



29 **334**

Scheme 2.19 Synthesis of dihydroerucalexin (**334**). Reagents and conditions: (i) $NaBH_4$, MeOH, 0 °C, 60%;



Scheme 2.20 Synthesis of acetamides **340**, **344**, **348** and **349**. Reagents and conditions: (i) Pyr, Ac_2O, DCM, 0 °C, 74-90%.

2.1.4.2 Antifungal activity

To determine if compounds **29**, **96**, **111**, **153**, **320**, **325**, **327**, **331**, **333** and **342** were metabolized by *L. maculans* through detoxification pathways, the antifungal activities of metabolites **95**, **155**, **156**, **324**, **326**, **330**, **334**, **339**-**341**, **343**, **344** and **346**-**349** against *L. maculans* were determined using the mycelia radial growth bioassay reported in the experimental (Pedras and Jha, 2006). The results are averages of experiments

conducted in triplicates, at least twice. As seen from several examples in section **2.1.3.1** and **2.1.3.2**, *L. maculans* is capable of metabolizing many of the antifungal compounds.

Comparison of the antifungal activities suggested that compound **29** (0.50 mM) showed about 40% inhibition of fungal growth, while compound **334** (0.50 mM) showed only about 23% inhibition. Thus, metabolism of compound **29** (Scheme **2.7**) was identified as a detoxification process in *L. maculans*. It is worth highlighting that compound **29** is known to inhibit BO, a detoxifying enzyme involved in detoxification of brassinin (**17**) (Pedras, Minic et al., 2008a).

Further, antifungal activities of compound **111** and metabolites **339** and **340** against *L. maculans* (BJ-125) were compared. The results established that the inhibitory activity decreased as follows: **111** > **339** > **340** (Table **2.1**). Since the metabolites **339** and **340** were less antifungal than compound **111,** metabolism of compound **111** by *L. maculans* (BJ-125) was a detoxification process. A metabolic detoxification of compound **111** was earlier reported in *S. sclerotiorum* (Pedras and Hossain, 2006), although to a different product. Similarly, antifungal assays of compound **342** and metabolites **343** and **344** against *L. maculans* (BJ-125) established the decreasing order of inhibitory activity as follows: **342** > **343** > **344** (Table **2.1**). Thus, metabolism of compound **342** by *L. maculans* (BJ-125) (Scheme **2.12**) was also determined to be a detoxification process. Further, the antifungal activity of compound **96** with those of metabolites **155** and **345-347** was compared. The higher antifungal activity of compound **96** compared to its products **155** and **345-347** confirmed that its metabolism is a detoxification process, as reported earlier (Pedras and Okanga, 2000). Next, the products of **325** metabolism by *L. maculans*, **95**, **324** and **348** displayed their antifungal activities in the following order: **95** < **348** < **324**, suggesting a detoxification process. Further, the antifungal activities of the nonindolyl dithiocarbamates and their metabolic products followed a similar trend as above, indicating their detoxification by *L. maculans*. This was evident from the relative antifungal activity of each compound, **327** > **326**, and **331** > **349**.

Finally, the products from the metabolism of dithiocarbonates **153** and **333**, **156** and **332** respectively, displayed lower antifungal activities than the parent compounds. On

this basis, it was concluded that the metabolism of indolyldithiocarbonates **153** and **333** is a detoxification. Antifungal activities of the respective transformation products **95**, **155**, **156**, **324**, **326**, **330**, **332**, **334**, **339**-**341**, **343**, **344** and **346**-**349** against *L. maculans* are summarized in table **2.2**.

Table 2.2 Percentage of growth inhibition[a] of *Leptosphaeria maculans* incubated with metabolites **95**, **155**, **156**, **324**, **326**, **330**, **332**, **334**, **339-341**, **343**, **344** and **346-349.**

Compound	Inhibition ± SD (%)[a]		
	0.50 mM	0.20 mM	0.10 mM
3-(3-Indolyl)propanoic acid (**95**)	78 ± 2	67 ± 4	41 ± 5
Tryptamine (**155**)	28 ± 2	15 ± 5	12 ± 3
Tryptophol (**156**)	28 ± 5	16 ± 4	n.i.
3-Indolylpropanamine (**324**)	27 ± 5	14 ± 4	n.i.
Benzimidazole (**326**)	45 ± 7	28 ± 4	15 ± 2
4-Biphenyl methanamine (**330**)	n.s.	n.s.	n.s.
3-(3-Indolyl)-1-propanol (**332**)	18 ± 2	14 ± 3	7 ± 2
Dihydroerucalexin (**334**)	23 ± 2	13 ± 2	7 ± 2
2-Indolylmethanamine (**339**)	33 ± 2	19 ± 4	n.i
N_b-Acetyl-2-indolylmethanamine (**340**)	33 ± 4	18 ± 2	7 ± 2
Indole-2-carboxylic acid (**341**)	19 ± 2	7 ± 2	n.i
1-Methoxy-2-indolylmethanamine (**343**)	19 ± 3	8 ± 3	n.i
N_b-Acetyl-1-methoxy-2-indolylmethanamine (**344**)	30 ± 3	11 ± 3	4 ± 2
N_b-Acetyltryptamine (**346**)	15 ± 3	7 ± 2	n.i
Indole-3-acetic acid (**347**)	32 ± 3	17 ± 5	10 ± 3
N_b-Acetyl-3-3-(3-indolyl)propanamine (**348**)	24 ± 3	13 ± 4	10 ± 3
N-Acetyl-4-biphenylmethanamine (**349**)	30 ± 5	14 ± 4	9 ± 4

[a] The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth on amended/growth in control) × 100]; n.i.= no inhibition; n.s.= not soluble.

2.1.5 Conclusions

In summary, the metabolic pathways of brassinin analogs dithiocarbamates **96**, **111**, **320**, **325**, **327**, **331**, **342** and dithiocarbonates **153, 333** appear to be similar, involving hydrolysis as the key step followed by further transformations. Rates of metabolism of the compounds **29**, **96**, **111**, **153**, **320**, **325**, **327**, **331**, **333** and **342** compared to compound **17** were different; there was no correlation between the metabolic rates and the antifungal activity of the compounds as previously observed in *S. sclerotiorum* (Pedras and Hossain, 2006). Slower metabolic rates of compound **342** relative to **320**, compound **96** relative to **325**, and compound **153** relative to **333** might suggest that the hydrolase(s) likely catalyzing these transformations are not very selective and accept a wide range of substrates. Metabolites **95**, **155**, **156**, **324**, **326**, **330**, **334**, **339-341**, **343**, **344** and **346-349** displayed lower antifungal activities against *L. maculans* compared to their respective parent compounds **29**, **96**, **111**, **153**, **320**, **325**, **327**, **331**, **333** and **342**. Thus, metabolism of the phytoalexin analogs **96**, **111**, **153**, **320**, **325**, **327**, **331**, **333**, **342** and the phytoalexin **29** were identified as detoxification reactions by *L. maculans*. Rapalexin A (**22**) was found to be resistant to metabolism by *L. maculans*. Results of these biotransformation experiments are summarized below in table **2.3**.

Table 2.3 Products of metabolism in cultures of *Leptosphaeria maculans* incubated with phytoalexins **17**, **22**, **29**, **36** and analogs **96**, **111**, **153**, **320**, **325**, **327**, **331**, **333** and **342**.

Compound incubated with cultures/ Antifungal activity (%)[b] at 0.50 mM	Recovery at 24 h	Major products of metabolism, molar %[a] (time in h) / Antifungal activity (%)[b] at 0.50 mM
Brassinin (**17**)/ 55 ± 3	< 5%	Indole-3-carboxaldehyde (**74**)
Rapalexin-A (**22**)/ c.i.	65%	No metabolism
Erucalexin (**29**)/ 40 ± 4	n.d.	Dihydroerucalexin (**334**), 33% (48)/ 23 ± 2
Brussalexin (**36**)[c]/ 43 ± 3	< 50%[c]	Not stable[d]
Methyl trytaminedithiocarbamate (**96**)/ c.i.	55%	Methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (**345**), 38% (72)/ 5 ± 1
		*N*b-Acetyltryptamine (**346**), 20% (72)/ 15 ± 3
Isobrassinin (**111**)/ 44 ± 4	< 5%	*N*b-Acetyl-2-indolylmethanamine (**340**), 30% (48)/ 33 ± 4
Tryptophol dithiocarbonate (**153**)/ 30 ± 3	15%	Tryptophol (**156**), 32% (24)/ 28 ± 5
Methyl indolyl-3-methylcarbamodithioate (**320**)[c]/ 61 ± 5	~30%[c]	Not stable[d]
Methyl 3-(3-indolyl)propyldithiocarbamate (**325**)/ 67 ± 3	37%	*N*b-Acetyl-3-(3-indolyl)propanamine (**348**), 6% (24)/ 24 ± 3
Methyl benzimidazolyl dithiocarbamate (**327**)/ c.i.	10%	Benzimidazole (**326**), 62% (24)/ 45 ± 7
Methyl 4-biphenyl dithiocarbamate (**331**)/ 46 ± 4	< 5%	*N*-Acetyl-4-biphenylmethanamine (**349**), 21% (72)/ 30 ± 5
Methyl 3-(3-indolyl)propyldithiocarbonate (**333**)/ 32 ± 4	n.d.	3-(3-Indolyl)-1-propanol (**332**), 60% (24)/ 18 ± 2
1-Methoxyisobrassinin (**342**)/ 34 ± 3	47%	*N*b-Acetyl-1-methoxy-2-indolylmethanamine (**344**), 37% (48)/ 30 ± 3

[a] Percentages were determined using a calibration curve and are average of experiments conducted in triplicates ± standard deviation; [b] percentages were determined using the formula: % inhibition = 100 – [(growth on amended/growth in control) × 100]; [c] compounds were tested in media alone and found not stable; [d] degraded to 3-indolylmethanol (**337**); c.i. = complete inhibition; n.d. = not detected.

2.2 Brassinin detoxification inhibitors

The design of inhibitors of brassinin detoxification is a challenging task. It took a great deal of work to identify inhibitors of BOLm. Among 80 compounds tested, analogs of brassinin (**17**) and related compounds (Appendix **A1D**), none inhibited BOLm activity (Pedras, Minic et al., 2008a). Screening of libraries of natural products and additional synthetic compounds (Section **1.4.3**) led to the identification of the first inhibitors of BOLm (Pedras, Jha et al., 2007c). Optimization of the lead structures, especially brassilexin (**30**) (Pedras, Minic et al., 2010a) and camalexin (**32**) (Pedras, Minic et al., 2009d) resulted in more potent inhibitors of BOLm (Appendix). Most of the known inhibitors of BOLm have indolyl or related moieties with relevant substitution at positions 2, 3, 5 and 6. It was shown that compound **168** containing a 2-naphthylyl moiety was also a potent inhibitor of BOLm (Pedras, Minic et al., 2009d). Since the additional number of compounds of interest that could be synthesized based on the indolyl and napthylyl moieties are limited, a replacement of these skeletons was looked for. Replacement of the heterocyclic moiety in the lead structure with a different hetero-aromatic unit is a general strategy explored in lead-optimization studies (Giardina, Artico et al., 1999). As seen in section **1.5**, a similar strategy was adopted to produce some of the agrochemicals discussed.

Here, it was thought to explore non-indolyl heteroaromatic systems in lead optimization studies. Assuming that indolyl and naphthylyl skeletons have sizes that are suitable for entry to the active site of BOLm, a hybrid of these moieties, i.e. a quinoline/ isoquinoline was chosen to produce potential inhibitors of BOLm. Two groups of potential inhibitiors were designed: (i) the first group of inhibitors containing thiazole and phenyl moieties attached to the substituted quinoline skeleton and (ii) the second group of inhibitors containing thiazole and phenyl moieties attached to the substituted isoquinoline skeleton (Figure **2.13**). Compounds **353**, **358**, **360**, **363**, **364** and **366** are new compounds. Next, the synthesis of the compounds **350**-**366** was undertaken, as described below.

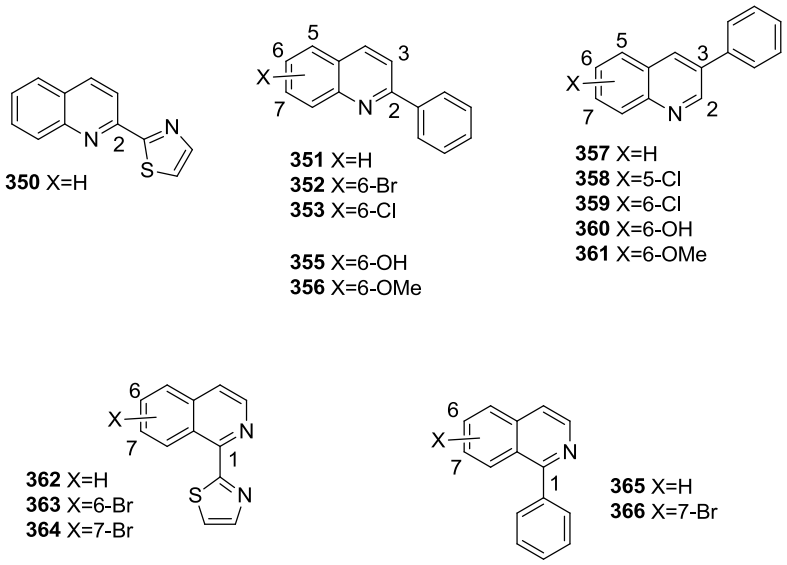


Figure 2.13 Potential brassinin detoxification inhibitors **350**-**366**.

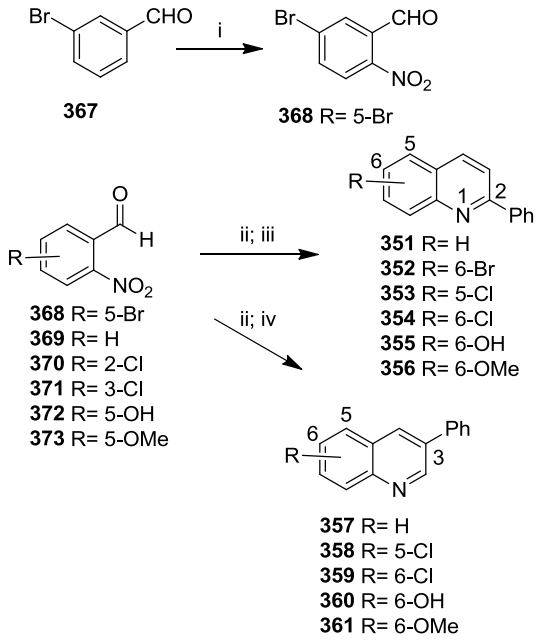
2.2.1 Synthesis

2.2.1.1 Synthesis of quinoline derivatives

Synthesis of **350** was carried out according to a published procedure (Dondoni, Dall' Occo et al., 1984) in comparable yield, as reported in the experimental.

Starting material **368** was prepared according to a reported procedure (Alford and Schofield, 1952). Nitration of 3-bromobenzaldehyde (**367**) with HNO_3/H_2SO_4 afforded the required product **368** (Scheme **2.21**). Synthesis of the 2-phenylquinolines **351**-**356** followed a general procedure (Li, Ahmed et al., 2007). Substituted 2-nitrobenzaldehydes **368**-**373** were reduced in the presence of Fe/HCl and the intermediates were treated with acetophenone in presence of KOH to afford the desired compounds **351**-**356** (Scheme **2.21**). This reaction can be applied to wide range of substrates and is probably a more efficient way to obtain 2-phenylquinolines than the alternative routes involing cross-coupling reactions (Vuoti, Autio et al., 2008). Synthesis of the 3-phenylquinolines **357**-**361** followed a general procedure (Li, Ahmed et al., 2007). Similarly, substituted 2-nitrobenzaldehydes **369**-**373** were reduced in the presence of Fe/HCl and the intermediates were treated with phenylacetaldehyde in presence of KOH to afford the desired compounds **357**-**361** (Scheme **2.21**). Reactions

involving the substrate **372** leading to the products **355** and **360** were low yielding, whereas similar reactions involving the substrate **373** leading to compounds **356** and **361** afforded higher yields. Overall, the one-pot reactions yielded the 2- and 3-phenyl substituted quinolines in moderate to good yields.

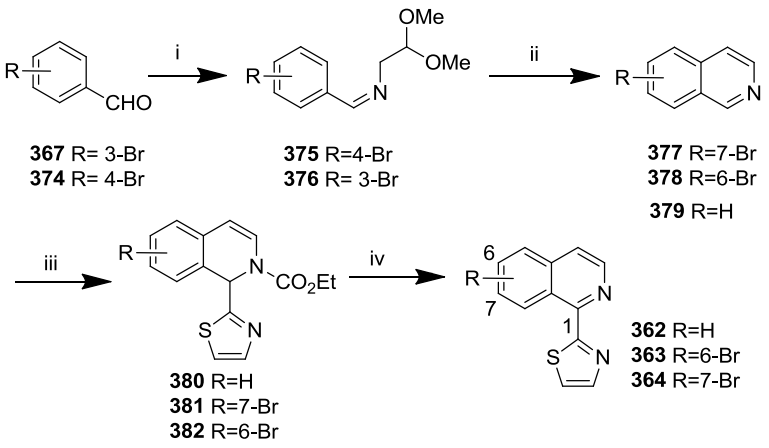


Scheme 2.21 Synthesis of compounds **351-361**. Reagents and conditions: (i) HNO_3/H_2SO_4, 0-5 °C, 68% (Alford and Schofield, 1952); (ii) Fe/HCl, EtOH; (iii) KOH, acetophenone, 42-83%; (iv) KOH, $PhCH_2CHO$, 39-80%.

2.2.1.2 Synthesis of isoquinoline derivatives

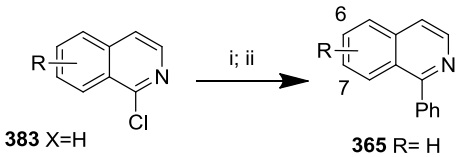
Intermediates **377** and **378** were prepared by a modification of the Pomeranz-Fritsch reaction used for preparation of isoquinolines (Jiang, Duckett et al., 2007). Substituted benzaldehydes **367** and **374** were treated with aminoacetaldehyde dimethyl acetal (Dean-Stark conditions) and the imine acetal intermediates **375** and **376** were cyclized in the presence of H_2SO_4 and P_2O_5 to yield substituted isoquinolines **377** and **378**. The reaction yields over two steps were in the range 10-20% and were lower than those reported earlier (Jiang, Duckett et al., 2007; Czako, Kurti et al., 2009). Finally, synthesis of compounds **362-364** was carried out starting from compounds **377-379**

using the same procedure adopted for the synthesis of compound **362** (Dondoni, Dall' Occo et al., 1984) (Scheme **2.22**).



Scheme 2.22 Synthesis of compounds **362-364.** Reagents and conditions: (i) aminoacetaldehyde dimethyl acetal, toluene, reflux (Dean-Stark); (ii) P_2O_5, H_2SO_4, 160 °C, 15-20% (Czako, Kurti et al., 2009); (iii) $ClCO_2Et$, DCM, 2-trimethylsilylthiazole, r.t.; (iv) *o*-chloranil, toluene, reflux, 15-32%.

Starting material **383** was synthesized according to a reported procedure (Alcock, Wirth et al., 1993) and compound **384** was purchased from Alfa Aesar. Syntheses of 1-phenylisoquinolines **365** and **366** were carried out using modification of a general procedure (Korn, Schade et al., 2006). Substituted 1-chloroisoquinolines **383** and **384** were treated with iodine followed by PhMgCl and Fe powder in THF to afford the desired compounds **365** and **366**. Addition of iodine to the reaction mixture was an extra step towards the modification of the reported procedure, which failed to yield the product. Ideal conditions for better yields employed the following stoichiometry of chemicals: substituted 1-chloroisoquinoline (1eq), iodine (1 eq), PhMgCl (1.2 eq), and Fe powder (2 eq) (Scheme **2.23**). On the other hand, 1-phenylisoquinolines can also be prepared by cross-coupling reactions (Jin, Gu et al., 2011; Tian, Lenkeit et al., 2010).



383 X=H
384 R= 7-Br

365 R= H
366 R= 7-Br

Scheme 2.23 Synthesis of compounds **365** and **366**. Reagents and conditions: (i) I_2, THF; (ii) PhMgCl, Fe, THF (Korn, Schade et al., 2006).

2.2.2 Antifungal activity

The antifungal activity of compounds **350**-**366** was determined against *L. maculans* (isolate BJ-125) using a mycelia radial growth bioassay as previously described. The results are the averages of experiments carried out in triplicates, at least twice. Results showed that compounds **355**, **357**, **361**, **362** and **364** displayed higher antifungal activity against *L. maculans* than brassinin (**17**). Among these, compounds **357**, **361** and **362** inhibited the blackleg fungal growth completely. Compounds **350**, **351**, **361** and **365** showed antifungal activity similar to that of brassinin (**17**) at 0.50 mM. Compounds **352**-**354**, **356**, **358** and **359** showed lower antifungal activity than brassinin (**17**) against *L. maculans*. There seemed to be no general trend regarding structure-activity relationship. Among substituted 3-phenylquinolines, compounds **358** and **359** with Cl substitution at position 5 and 6 of 3-phenylquinoline moiety displayed lower antifungal activities than the parent compound **357**. The antifungal activity of compounds **360** and **361** was higher than brassinin (**17**), but lower than that of the parent unsubstituted compound **357**. All the substituted 2-phenylquinolines were less inhibitory against *L. maculans* than the parent unsubstituted compound **351** and brassinin (**17**), except compound **355**. Compound **362** displayed higher antifungal activity than compound **350**. Compounds **363** and **364** with Br substitution at positions 6 and 7 of the 1-(thiazol-2-yl)isoquinoline (**362**) moiety displayed lower antifungal activities than the parent compound **362,** but both the compounds **363** and **364** were more inhibitory against *L. maculans* than brassinin (**17**). 7-Bromo-1-phenylisoquinoline (**366**) displayed less antifungal activity than compound **365.** Antifungal activities of the compounds **350**-**366** are summarized in table **2.4**.

Table 2.4 Percentage of growth inhibition[a] of *Leptosphaeria maculans* incubated with potential brassinin (**17**) detoxification inhibitors **350**-**366** (5 days, constant light).

Compounds	Inhibition ± SD (%)[a]		
	0.50 mM	0.20 mM	0.10 mM
Brassinin **(17)**	62 ± 4	25 ± 3	12 ± 4
2-(Thiazol-2-yl) quinoline (**350**)	54 ± 3	46 ± 4	8 ± 3
2-Phenylquinoline (**351**)	54 ± 2	31 ± 4	16 ± 5
6-Bromo-2-phenylquinoline (**352**)	27 ± 4	14 ± 4	n.i.
5-Chloro-2-phenylquinoline **(353)**	39 ± 2	18 ± 4	9 ± 2
6-Chloro-2-phenylquinoline **(354)**	26 ± 4	18 ± 5	5 ± 2
6-Hydroxy-2-phenylquinoline **(355)**	68 ± 5	42 ± 4	32 ± 4
6-Methoxy-2-phenylquinoline **(356)**	36 ± 4	20 ± 4	8 ± 2
3-Phenylquinoline **(357)**	c.i.	77 ± 4	39 ± 7
5-Chloro-3-phenylquinoline **(358)**	17 ± 3	11 ± 2	8 ± 4
6-Chloro-3-phenylquinoline **(359)**	28 ± 3	17 ± 3	5 ± 2
6-Hydroxy-3-phenylquinoline **(360)**	73 ± 5	45 ± 4	30 ± 4
6-Methoxy-3-phenylquinoline **(361)**	c.i.	68 ± 5	55 ± 5
1-(Thiazol-2-yl)isoquinoline (**362**)	c.i.	c.i.	52±4
6-Bromo-1-(thiazol-2-yl)isoquinoline (**363**)	75 ± 6	54 ± 3	15 ± 4
7-Bromo-1-(thiazol-2-yl)isoquinoline (**364**)	82 ± 5	63 ± 3	25 ± 6
1-Phenylisoquinoline (**365**)	54 ± 5	32 ± 4	30 ± 3
7-Bromo-1-phenylisoquinoline (**366**)	47 ± 3	31 ± 4	12 ± 2

[a] The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth on amended/growth in control) × 100]; c.i. = complete inhibition; n.i.= no inhibition.

2.2.3 Screening for inhibition of brassinin detoxification

Screening of potential inhibitors of brassinin detoxification can be performed using fungal cultures or the purified detoxifying enzyme, BOLm. Both studies are necessary for preliminary identification of inhibitors of brassinin detoxification. In particular, screening of the potential inhibitors in fungal cultures is important because it would give information about their effect on brassinin detoxification as well as the stability of compounds in vivo. Thus, results of screening studies in fungal cultures are useful in providing the information about interesting compounds that can be used in enzymatic screening. In the current thesis, studies related to screening of the potential inhibitors in fungal cultures are discussed.

Previously, brassinin (**17**) was co-incubated with compounds **153**, and **157-159** (0.1 mM) in 48 h-old cultures of *L. maculans* (10^6/ml) (Pedras and Jha, 2006). It was observed that brassinin (**17,** 0.10 mM) was metabolized at slower rates (> 15 h) in presence of compounds **153**, and **157-159** than in control cultures (0.10 mM, 15 h). It was hypothesized that slower metabolism of brassinin (**17**) in the presence of tested compounds could be due to: (i) inhibition of brassinin detoxification or/ and (ii) inhibition of fungal growth. Thus, knowledge of the antifungal activities of the potential inhibitors, and effect of these compounds on brassinin metabolism in fungal cultures was important for preliminary evaluation. On this basis, compounds **153**, **157-159**, that showed low inhibition of the growth of *L. maculans* but a significant effect in inhibition of brassinin detoxification, were considered as compounds of interest for paldoxin design (Pedras and Jha, 2006). Later on, in vitro screening using purified BOLm determined that compounds **153** and **157-159** were not inhibitors of BOLm (Pedras, Jha et al., 2007c).

On the other hand, camalexin (**32**), a phytoalexin which did not inhibit brassinin detoxification in fungal cultures (Pedras, Jha et al., 2005b), was determined to be an inhibitor of BOLm (Pedras, Jha et al., 2007c). Camalexin (**32**) was not identified as inhibitor of brassinin detoxification from screening studies in fungal cultures because compound **32** was both inducer and inhibitor of BOLm (Pedras, Minic et al., 2009c).

Because, induction and inhibition of BOLm occurred simultaneously, it was likely that the fungal culture conditions in which brassinin (**17,** 0.10 mM) was quickly metabolized (15 h) might not be discriminative. It was thought that slower rates of brassinin metabolism (detection up to 48 h instead of 15 h) may be required to detect the inhibitory effect of compounds like camalexin (**32**). Thus, better conditions for the screening of potential inhibitors of brassinin detoxification in fungal cultures were explored.

2.2.3.1 Optimization of screening method

Several experiments were carried out to optimize the screening conditions to specifically slow down the rate of brassinin metabolism in fungal cultures. Since the rate of brassinin metabolism is dependent on the amount of mycelia mass present in fungal cultures, conditions that stabilized the mycelia mass were first tried. It was predicted that addition of brassinin (**17**) and other inducers of BOLm to the fungal cultures in the exponential growth phase might induce the detoxifying enzyme of *L. maculans* rapidly, leading to faster metabolism of brassinin (**17**). Supportingly, it was observed that the rate of brassinin (**17**) metabolism was not decreased when exponentially growing mycelial cultures were co-incubated with camalexin (**32**), an inducer and inhibitor of BOLm (Pedras, Jha et al., 2005). On the other hand, it was observed that growth of the mycelia was inhibited if the toxic potential inhibitors and brassinin (**17**) were added to the fungal cultures in the exponential growth phase (Pedras and Jha, 2006). Thus, in order to establish a new screening protocol, culture conditions that do not allow rapid growth of the mycelia were employed.

Minimal media was inoculated with 10^6/ml spores and incubated for 7 d. After 7 d, mycelia were transferred to water and the cultures in water were incubated with brassinin (**17**, 0.1 mM) either immediately or after 24 h. In either case, metabolism of brassinin (**17**, 0.1 mM) was faster than in the original experiments (Pedras and Jha, 2006) (15 h); i.e. the compound was not detected after 6 h. Further, cultures of *L. maculans* (BJ-125, 10^4/ml) were grown in minimal media for 5 days and mycelia were transferred to water. After 3 days, brassinin (**17**) was incubated with mycelial cultures

in water. Under these conditions (Entry 7, table **2.5**), the brassinin (**17**) metabolism rate decreased (detected up to 48 h). Conditions tried towards optimization of the screening protocol (Entries 1-7) are summarized in table **2.5**.

Table 2.5 Culture conditions for optimization of the screening method to determine the effect of potential inhibitors on brassinin detoxification.

Entry	Glucose g/l	Spores #/ml	Incubation times (in MM)	Brassinin (0.10 mM) detection
1	15 g	10^6	2 d [a]	Up to 15 h
2	15 g	10^6	7 d → water (0 h)[b]	Up to 6 h
3	15 g	10^6	7 d → water (1 d)[b]	Up to 6 h
4	15 g	10^4	5 d → water (0 h)[b]	Up to 6 h
5	15 g	10^4	5 d → water (1 d)[b]	Up to 6 h
6	15 g	10^4	5 d → water (2 d)[b]	Up to 15 h
7	15 g	10^4	5 d → water (3 d)[b]	Up to 48 h

[a] Standard conditions: brassinin was added to 2 d-old mycelial cultures (Pedras and Jha, 2006); [b] mycelia from x d-old cultures (in MM) were transferred to water and incubated for additional x d before the addition of brassinin (0.10 mM).

In order to test the new conditions (Entry 7, table **2.5**), a camalexin analog, 5-methoxycamalexin (**167,** 72% inhibition of BOLm at 0.30 mM) (Pedras, Minic et al., 2009c) was tested for its inhibitory effect on brassinin detoxification. Mycelia from 5 d-old cultures in minimal media were transferred to water and, after three additional days, compound **167** (0.10 mM) followed by brassinin (**17**, 0.10 mM) were added and incubated up to three days. Samples were withdrawn at different time intervals, extracted with EtOAc, and the extracts were analyzed by HPLC-DAD. In the presence of compound **167 (**0.10 mM), complete metabolism of brassinin (**17**) was delayed until 96 h (vs ~48 h in control samples) (Figure **2.14**). In control cultures, the time required for 50% metabolism ($t_{1/2}$) of brassinin (**17**, 0.10 mM) was ~ 15 h and 100% metabolism (t_{Br}) of **17** was achieved after ~ 48 h. In the presence of compound **167**, values of $t_{1/2}$ and t_{Br} increased to 36 h and 96 h respectively, indicating the inhibition of brassinin detoxification in fungal cultures. Compound **167**, like compound **32**, is both an inducer and inhibitor of BOLm (Pedras, Minic et al., 2009c) and yet, the current screening protocol successfully demonstrated the decrease of brassinin detoxification rates in the presence of compound **167**. Thus, the new conditions (Entry 7, table **2.5**) proved

advantageous over previous conditions (Entry 1, table **2.5**) and would provide a better opportunity to observe the effect of potential inhibitors in decreasing the rate of brassinin detoxification over a long period (at least up to 48 h).

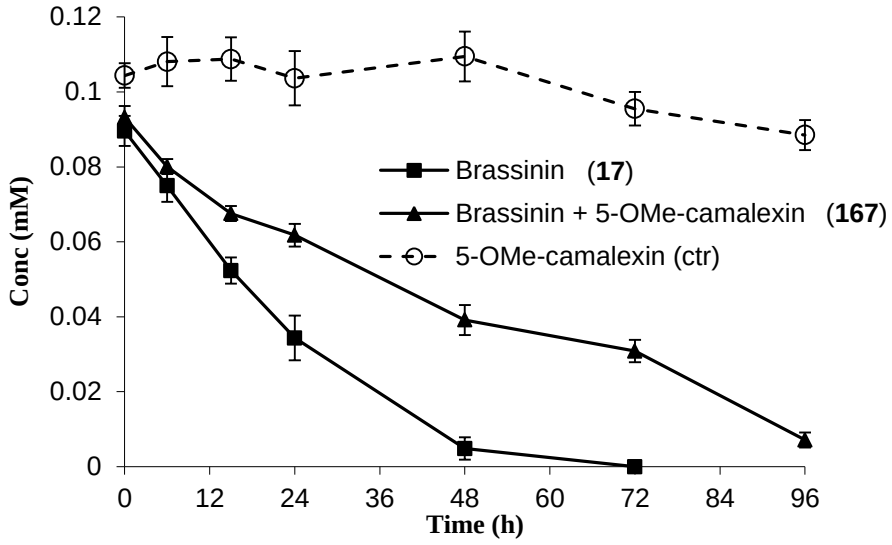


Figure 2.14 Metabolism of brassinin (**17**, 0.10 mM) in the presence of 5-methoxycamalexin (**167**, 0.10 mM) in cultures of *Leptosphaeria maculans* (10^4/ml spores).

2.2.3.2 Screening of potential inhibitors of brassinin detoxification

Each of the synthetic potential inhibitors was screened for the effect in decreasing the rate of brassinin detoxification according to the new screening method. In control cultures, time required for 50% metabolism ($t_{1/2}$) of brassinin (**17**, 0.10 mM) varied between 6-15 h and 100% metabolism (t_{Br}) of **17** was ~ 48 h. Higher values of $t_{1/2}$ and t_{Br} in the cultures of potential inhibitors co-incubated with brassinin (**17**) in comparison with those in controls are indicative of the effect of the tested compounds in decreasing the rates of brassinin detoxification. However, in some cases $t_{1/2}$ of brassinin in the presence of potential inhibitor is about the same as in control cultures (~ 6 h), but the values of t_{Br} were > 48 h. Among the quinoline derivatives, 2-thiazolylquinoline (**350,** 0.10 mM) did not affect the rate of brassinin metabolism (Figure **2.15**). In presence of compound **350,** complete metabolism of brassinin (**17**) took place in 48 h ($t_{Br)}$ and 50%

metabolism of compound **17** in 6 h ($t_{1/2}$). The values of $t_{1/2}$ and t_{Br} are similar to that of control cultures (incubated with only brassinin). Next, compounds **352**, **354** and **359** were co-incubated with brassinin (**17**, 0.10 mM). Comparison of the values of $t_{1/2}$ and t_{Br} in the cultures of compounds **352**, **354** and **359** co-incubated with brassinin (**17**, 0.10 mM) with those in the control cultures (incubated with only **17**) demonstrated that compound **354** did not affect the brassinin detoxification, while compound **359** decreaesd the brassinin metabolism rate to a small extent. Importantly, compound **352** upon co-incubation with brassinin (**17**) decreased its metabolism to a large extent. The value of $t_{1/2}$ was larger (~ 65 h vs 6 h in control) in the cultures of compound **352** co-incubated with brassinin (**17**) than in similar cultures of compounds **354** (~ 6 h vs 6 h in control) and **359** (~ 6 h vs 6 h in control). Brassinin metabolism in the presence of **352** was delayed to 96 h (Figure **2.16**).

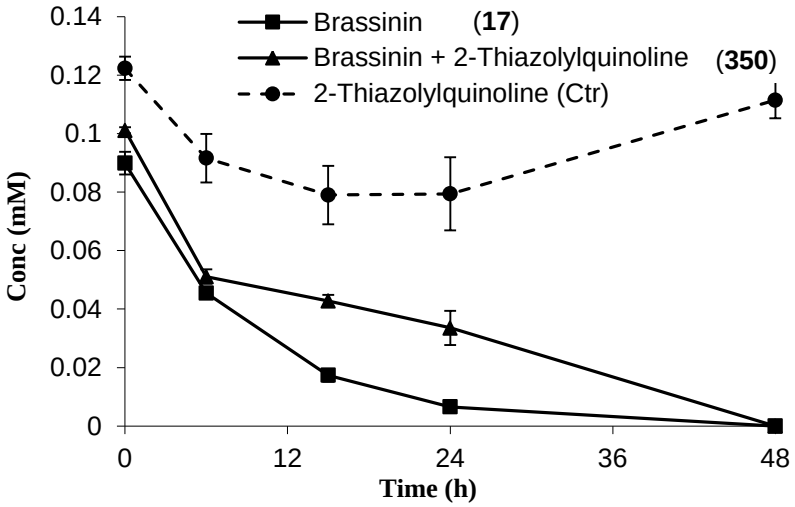


Figure 2.15 Progress curve for the detoxification of brassinin (**17**, 0.10 mM) in the presence of 2-(thiazol-2-yl) quinoline (**350**, 0.10 mM) in mycelia cultures of *Leptosphaeria maculans* in water.

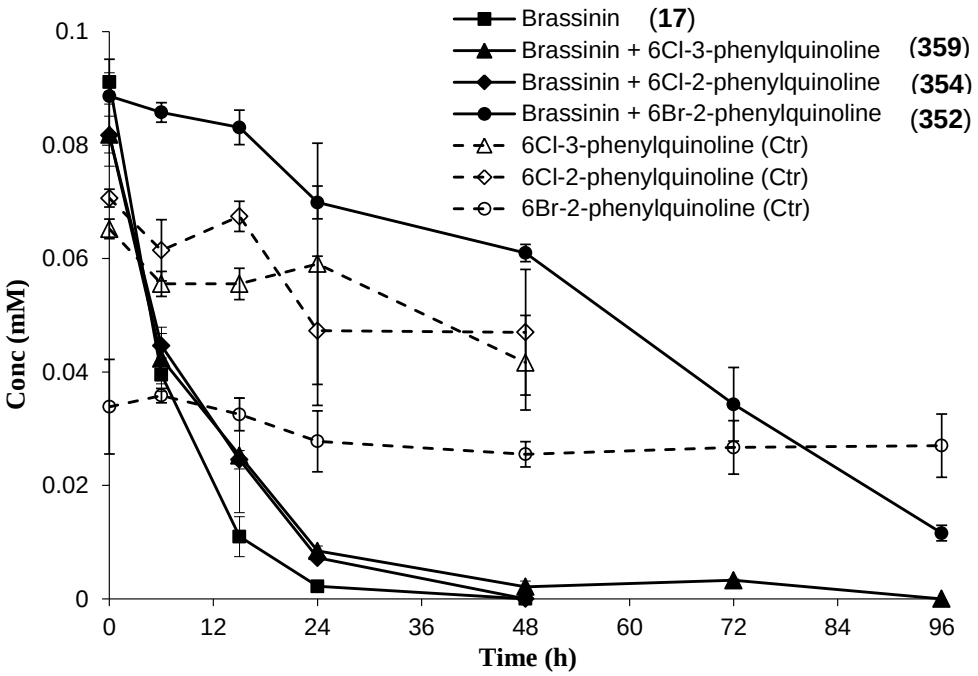


Figure 2.16 Progress curves for the detoxification of brassinin (**17**, 0.10 mM) in the presence of 6-bromo-2-phenylquinoline **(352**, 0.10 mM**)**, 6-chloro-2-phenylquinoline **(354**, 0.10 mM**)** and 6-chloro-3-phenylquinoline **(359**, 0.10 mM) in cultures of *Leptosphaeria maculans* in water.

Compounds **353** or **358** (0.10 mM) incubated with brassinin (**17**) had a moderate effect on its metabolic rate. Results indicated that brassinin (**17**) in the presence of **353** and **358** was completely metabolized in 72 h (48 h in control cultures, Figure **2.17**). The values of $t_{1/2}$ were greater (~ 24 h vs 6 h in control) in cultures incubated with brassinin (**17**) and containing compounds **353** or **358**, indicating their moderate effect in decreasing the rate of brassinin detoxification.

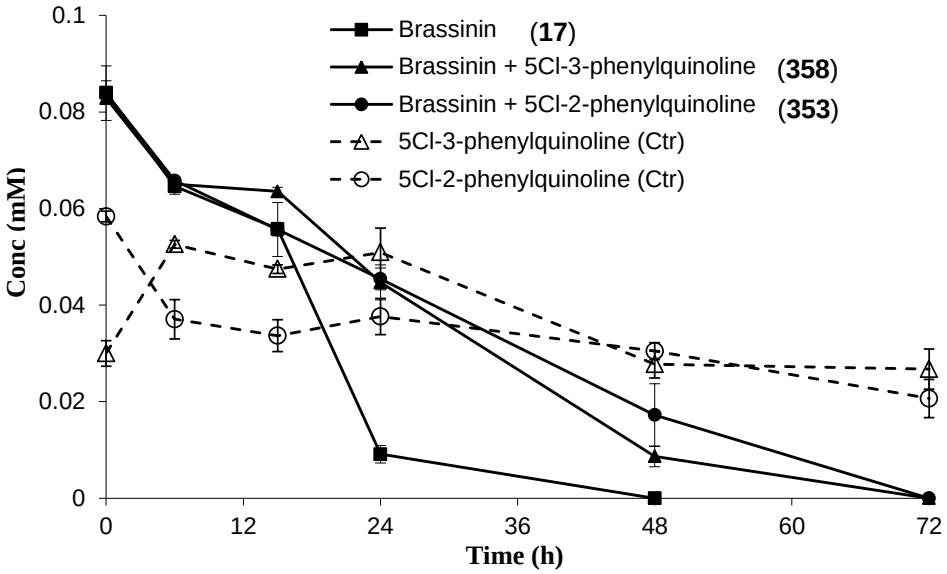


Figure 2.17 Progress curves for the detoxification of brassinin (**17**, 0.10 mM) in the presence of 5-chloro-2-phenylquinoline (**353**, 0.10 mM) and 5-chloro-3-phenylquinoline (**358**, 0.10 mM) in cultures of *Leptosphaeria maculans* in water.

Similarly, when compounds **355, 356, 360** and **361** were co-incubated with brassinin (**17**), all the compounds except **361** showed a small effect in decreasing the rate of brassinin detoxification. Brassinin (**17**, 0.10 mM) was completely metabolized in control cultures incubated with only brassinin and in cultures co-incubated with compound **361** within 48 h. Metabolism of brassinin was delayed up to 72 h when co-incubated with compound **356** (Figure **2.18**)**.** However, the values of $t_{1/2}$ in cultures of compounds **356** or **361** co-incubated with brassinin (**17**) were comparable to the $t_{1/2}$ value in control cultures. Metabolism of brassinin (**17**) was delayed up to 72 h when co-incubated with compound **355** and **360** (Figure **2.19**). However, the values of $t_{1/2}$ in cultures of compounds **355** and **360** co-incubated with brassinin (**17**) were comparable to the $t_{1/2}$ value in control cultures.

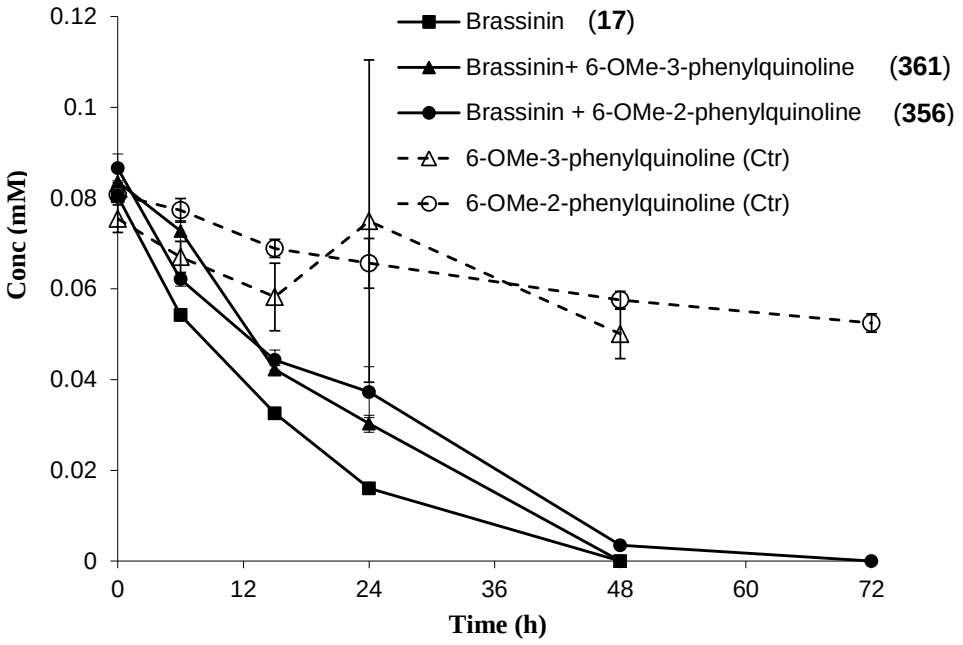


Figure 2.18 Progress curves for the detoxification of brassinin (**17**, 0.10 mM) in the presence of 6-methoxy-2-phenylquinoline (**356**, 0.10 mM) and 6-methoxy-3-phenylquinoline (**361**, 0.10 mM) in cultures of *Leptosphaeria maculans* in water.

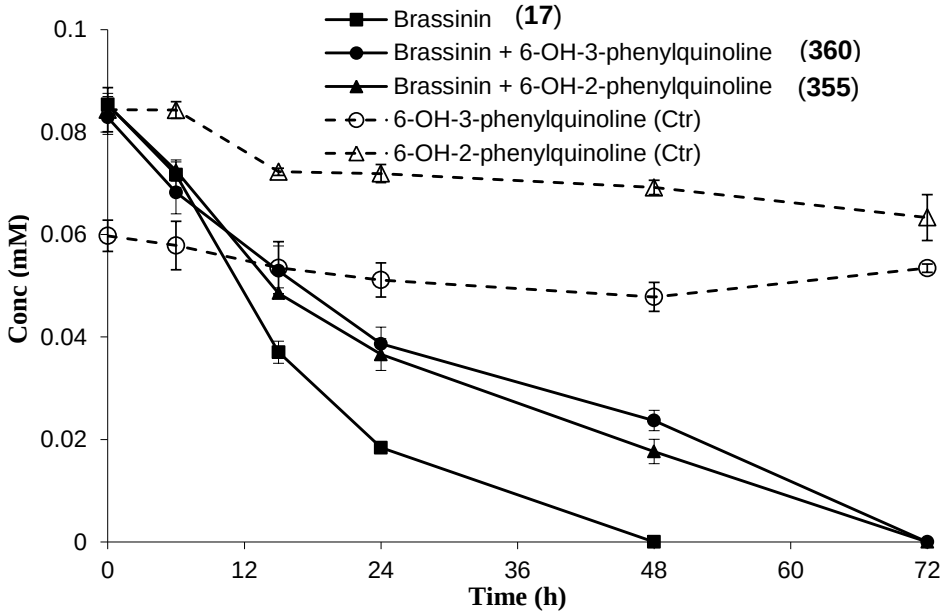


Figure 2.19 Progress curves for the detoxification of brassinin (**17**, 0.10 mM) in the presence of 6-hydroxy-2-phenylquinoline (**355**, 0.10 mM) and 6-hydroxy-3-phenylquinoline (**360**, 0.10 mM) in cultures of *Leptosphaeria maculans* in water.

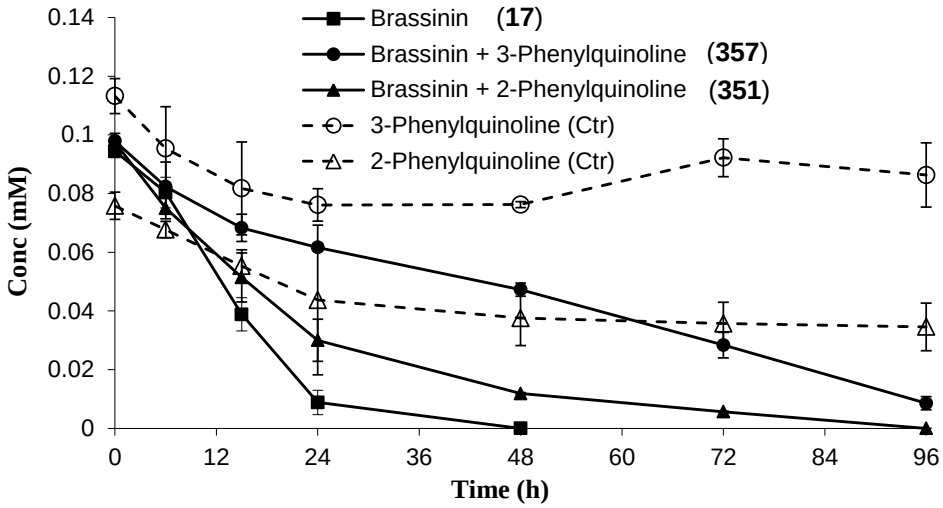


Figure 2.20 Progress curves for the detoxification of brassinin (**17**, 0.10 mM) in the presence of 2-phenylquinoline (**351**, 0.10 mM) and 3-phenylquinoline (**357**, 0.10 mM) in cultures of *Leptosphaeria maculans* in water.

The effect of compounds **351** and **357** (0.10 mM) on the rate of brassinin metabolism is shown in figure **2.20**. Both the compounds showed a substantial effect in decreasing the rates of brassinin metabolism. Complete metabolism of brassinin (**17**, 0.10 mM) took place at 96 h in presence of **351** and **357**, whereas in the absence of these compounds brassinin (**17**) was metabolized within 48 h. However, the value of $t_{1/2}$ in cultures of compounds **351** co-incubated with brassinin (**17**) was comparable to the $t_{1/2}$ value in control cultures. However, the value of $t_{1/2}$ in cultures of compound **357** co-incubated with brassinin (**17**) was ~ 48 h, suggesting a strong effect of compound **357** in decreasing the rate of brassinin detoxification.

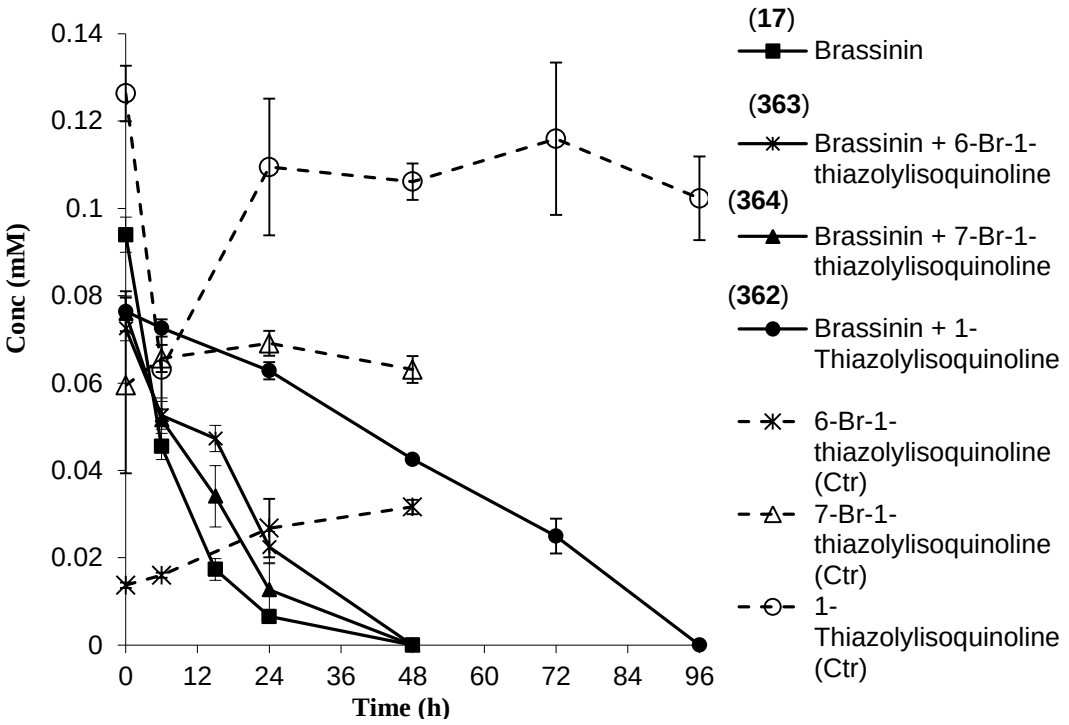


Figure 2.21 Progress curves for the detoxification of brassinin (**17**, 0.10 mM) in the presence of 1-(thiazol-2-yl)isoquinoline (**362**, 0.10 mM), 6-bromo-1-(thiazol-2-yl)isoquinoline (**363**, 0.10 mM) and 7-bromo-1-(thiazol-2-yl)isoquinoline (**364**, 0.10 mM) in cultures of *Leptosphaeria maculans* in water.

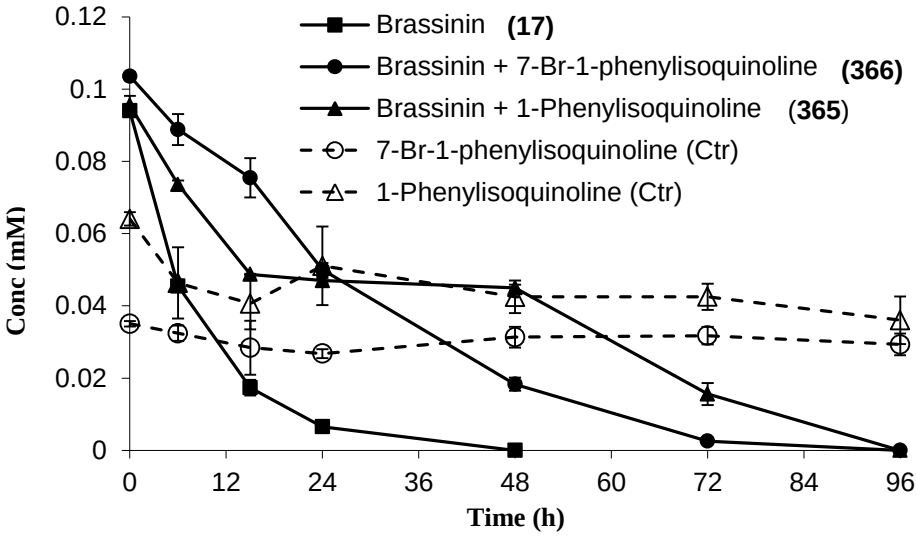


Figure 2.22 Progress curves for the detoxification of brassinin (**17**, 0.10 mM) in the presence of 1-phenylisoquinoline (**365**, 0.10 mM) and 7-bromo-1-phenylisoquinoline (**366**, 0.10 mM) in cultures of *Leptosphaeria maculans* in water.

Among the isoquinoline analogs **362**-**366**, compounds **363** and **364** did not affect the rate of brassinin metabolism when co-incubated with compound **17** in cultures of *L.*

maculans (BJ-125). Under these conditions, brassinin (**17**, 0.10 mM) in presence of compounds **363** and **364** was metabolized as in control cultures within 48 h. Even the values of $t_{1/2}$ in cultures of compounds **363** and **364** incubated with brassinin (**17**) were comparable to the $t_{1/2}$ value in control cultures. Metabolism of brassinin (**17**) was delayed up to 96 h when co-incubated with compound **362** (Figure **2.21**)**.** The value of $t_{1/2}$ in cultures of compounds **362** co-incubated with brassinin (**17**) was greater (45 h vs ~ 6 h) than the $t_{1/2}$ value in control cultures. This suggested that compound **362** has a significant effect in decreasing the rate of brassinin detoxification.

Compounds **365** and **366** appeared to have significant effect in decreasing the rate of brassinin detoxification. When the compounds **365** and **366** were co-incubated with brassinin (**17**), metabolism of **17** was delayed up to 96 h (Figure **2.22**). The values of $t_{1/2}$ in cultures of compounds **365** and **366** incubated with brassinin (**17**) were greater than those in control cultures, suggesting an effect of these compounds on the rate of brassinin detoxification.

However, in the above screening experiments (Figures **2.15-2.22**), from the recovery of potential inhibitors **350**-**366** from fungal cultures (shown as control curves), it was difficult to determine if the compounds were stable and not metabolized. Moreover, in these plots, compounds **350**-**366** were quantified based on the calibration curves not built at the time of the screening experiments but, at a later stage. On further comparison of the calibration curves of brassinin (**17**) built at the time of screening experiments and at a later stage, a discrepancy of approximately 20% was noted. Thus, the concentration of the compounds **350-366** from screening experiments shown in the graphs (Figures **2.15-2.22**) are potentially less than the reported values. It was not clear if the low recovery values of compounds **350**-**366** are due to the metabolism of the concerned compounds, especially because there were no metabolites detected in the fungal cultures (10^4 Spores/ ml). However, it would be possible that the compounds **350**-**366** might metabolize to metabolites that are not extractable from the fungal cultures. For further clarification, investigation of the metabolism of compounds **350**-**366** in fungal cultures (10^6 Spores/ml) was carried out in minimal media.

2.2.3.3 Metabolism of compounds 350-366

To establish if any of compounds **350**-**366** were metabolized by *L. maculans*, solutions in ACN/DMSO (final conc 0.10 mM) were added to fungal cultures in minimal media (10^6 spores/ml of *L. maculans*, isolates virulent on canola) and samples (2 ml) were collected after 0, 6, 24 and 48 h of incubation, extracted with EtOAc and analyzed by HPLC. Control experiments were also carried out by incubating compounds **350**-**366** in media alone.

The concentration of compounds **352**, **355**, **360**, **361**, **363** and **364** decreased in the fungal cultures, indicating that these compounds were rapidly metabolized (Figure **2.23**). Metabolites were detected only in the fungal cultures incubated with 6-hydroxy-2-phenylquinoline (**355**, t_R = 3.1 min) and 6-hydroxy-3-phenylquinoline (**360**, t_R = 3.3 min). Compounds **355** and **360** were completely metabolized to unknown metabolites within 48 h of incubation with fungal cultures. Compounds **351**, **353**, **356**, **358** and **359** were metabolized in the fungal cultures (Figure **2.24**) at a slow rate, although new metabolites were observed only from compounds **351** (t_R = 2.9 min) and **358** (t_R = 3.9 min). On the other hand, recoveries of compounds **350**, **354**, **357**, **362**, **365** and **366** from fungal cultures were found to be either very little or not at all changed over a 0-48 h period or showing large deviations, suggesting that these compounds might not be metabolized in fungal cultures (Figure **2.25**).

Altogether, comparison of the recoveries of compounds **350**-**366** in fungal cultures as well as in control media led to inconsistent results (Figures **2.23**-**2.25)**. Thus, further investigation is necessary to confirm the metabolism of these compounds.

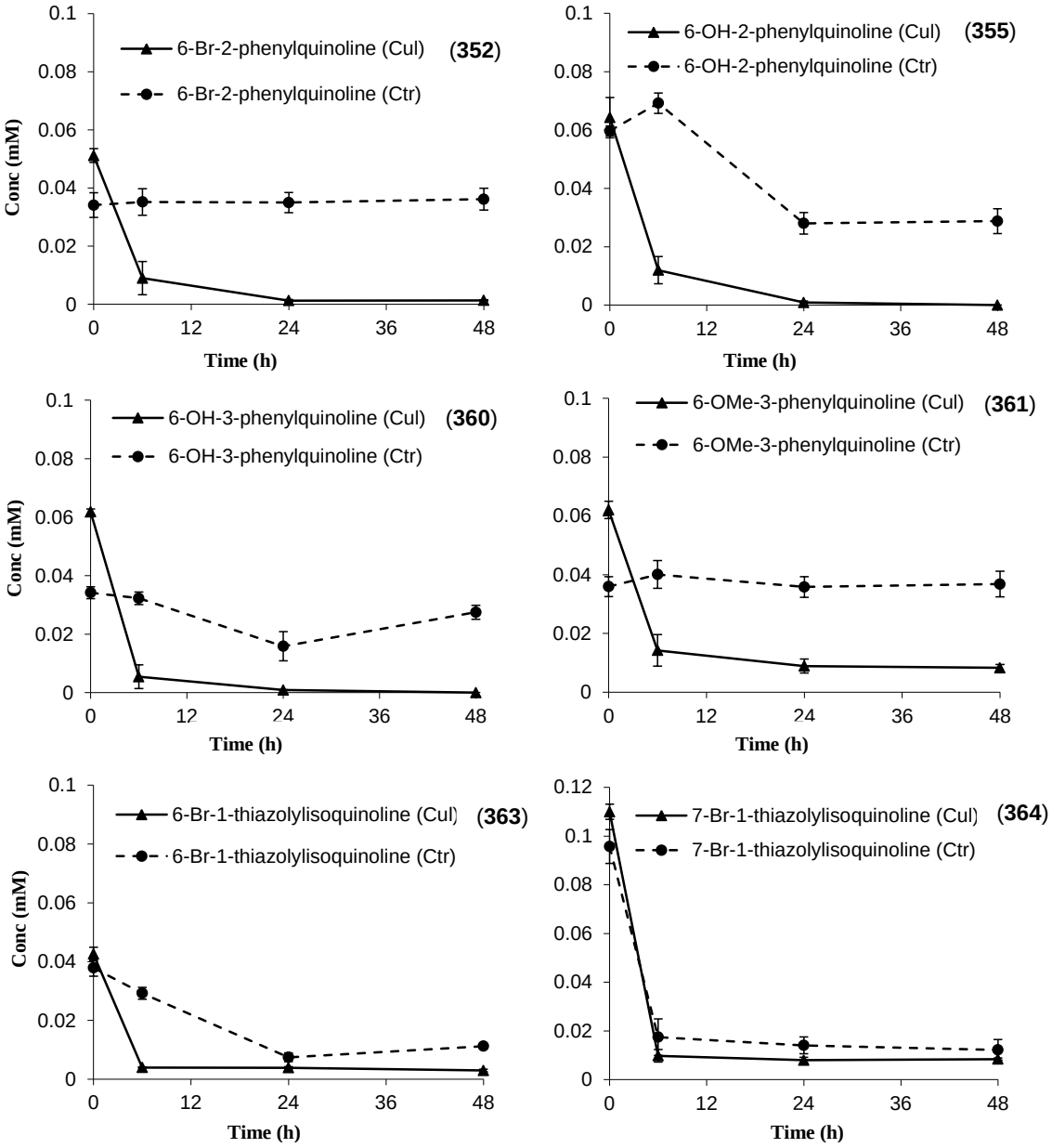


Figure 2.23 Progress curves representing the recovery of 6-bromo-2-phenylquinoline **(352**, 0.10 mM**),** 6-hydroxy-2-phenylquinoline (**355**, 0.10 mM), 6-hydroxy-3-phenylquinoline (**360**, 0.10 mM), 6-methoxy-3-phenylquinoline (**361**, 0.10 mM), 6-bromo-1-(thiazol-2-yl)isoquinoline (**363**, 0.10 mM) and 7-bromo-1-(thiazol-2-yl)isoquinoline (**364**, 0.10 mM) that seemed to metabolize rapidly in cultures (Cul) of *Leptosphaeria maculans*.

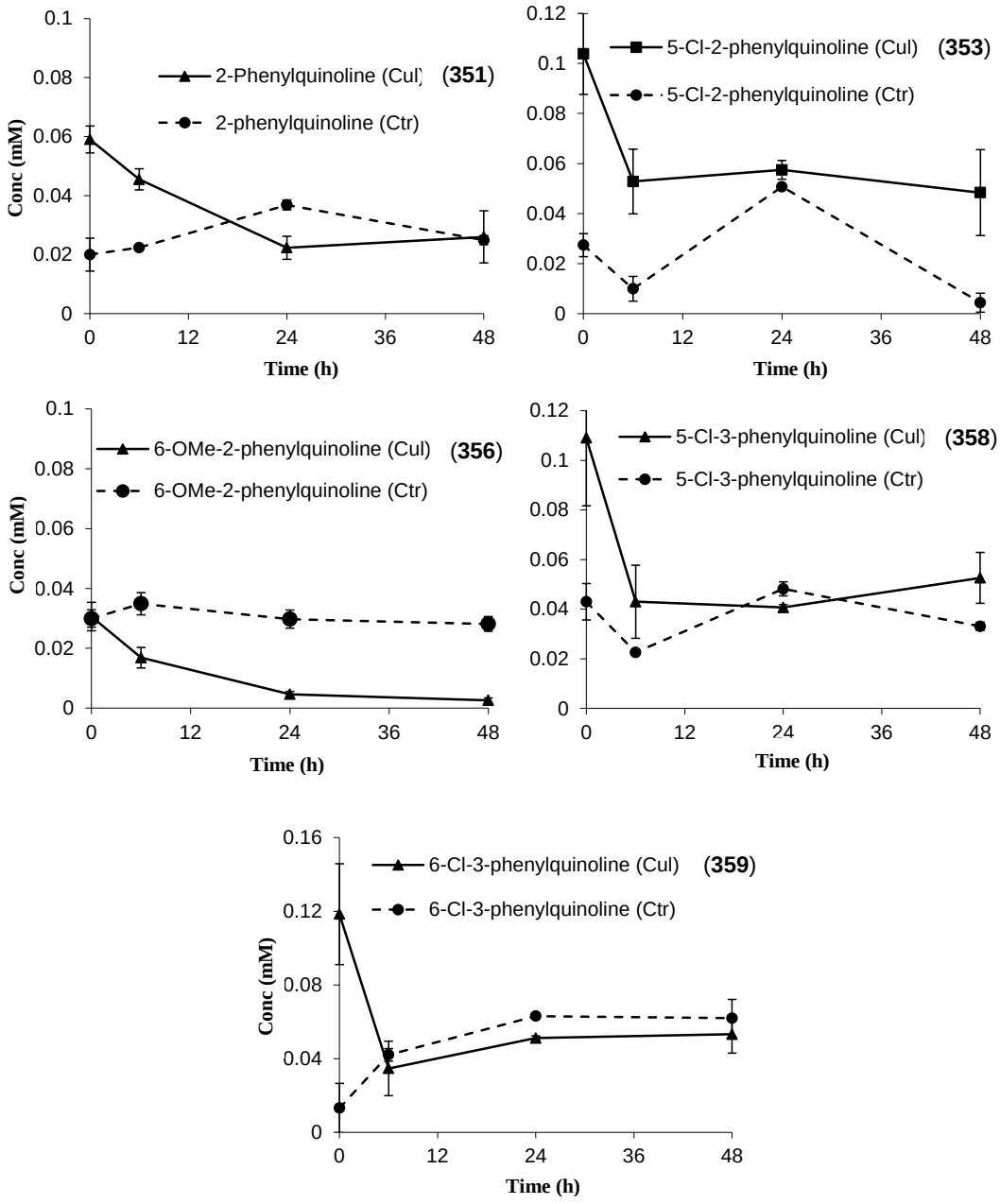


Figure 2.24 Progress curves representing the recovery of 2-phenylquinoline (**351**, 0.10 mM), 5-chloro-2-phenylquinoline (**353**, 0.10 mM), 6-methoxy-2-phenylquinoline (**356**, 0.10 mM), 5-chloro-3-phenylquinoline (**358**, 0.10 mM) and 6-chloro-3-phenylquinoline **(359**, 0.10 mM) that seemed to metabolize at a slow rate in cultures (Cul) of *Leptosphaeria maculans*.

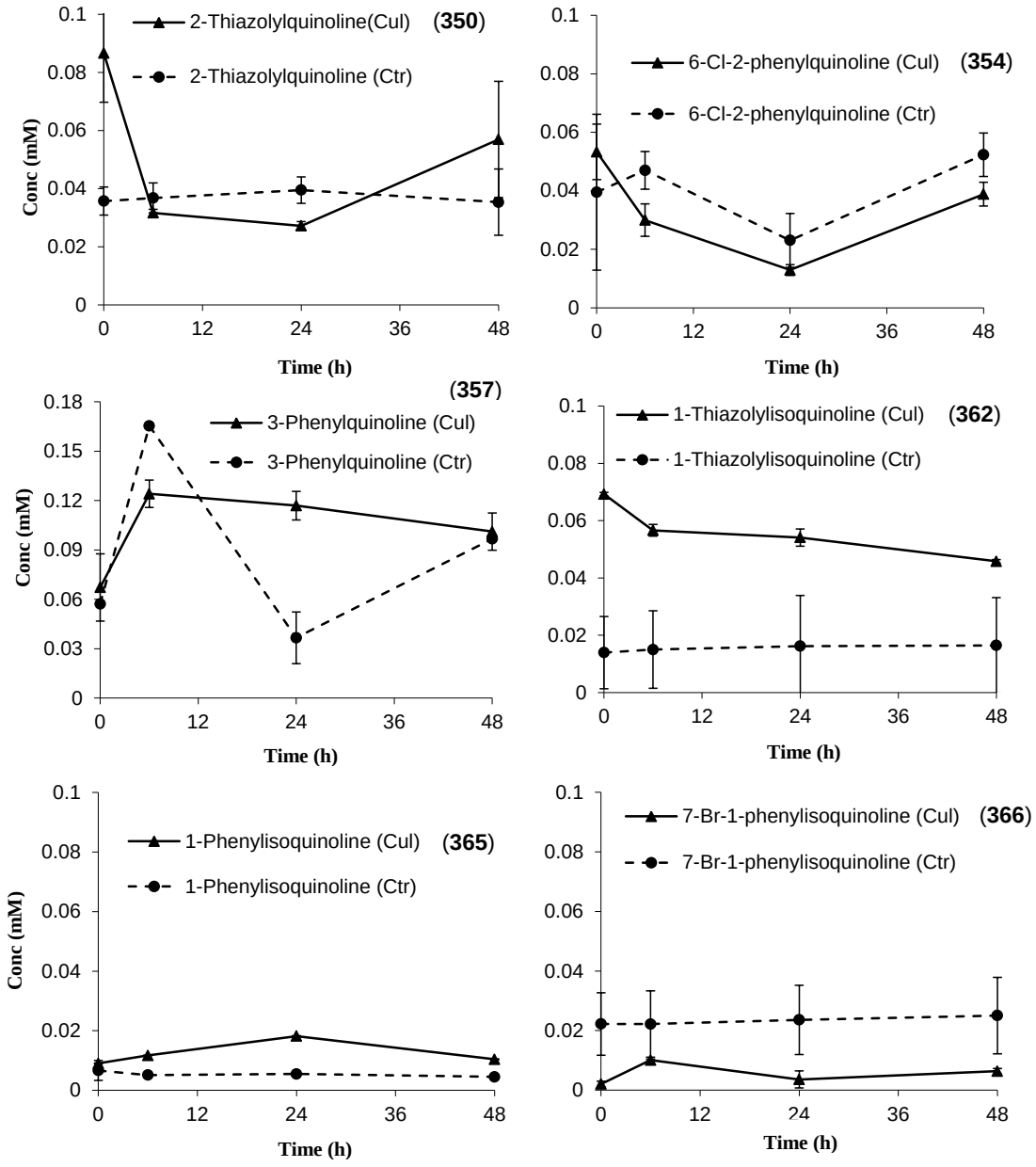


Figure 2.25 Progress curves representing the recovery of 2-(thiazol-2-yl) quinoline (**350**, 0.10 mM), 6-chloro-2-phenylquinoline **(354**, 0.10 mM**)**, 3-phenylquinoline (**357**, 0.10 mM), 1-(thiazol-2-yl)isoquinoline (**362**, 0.10 mM), 1-phenylisoquinoline (**365**, 0.10 mM) and 7-bromo-1-phenylisoquinoline (**366**, 0.10 mM) that are not likely metabolized in cultures (Cul) of *Leptosphaeria maculans*.

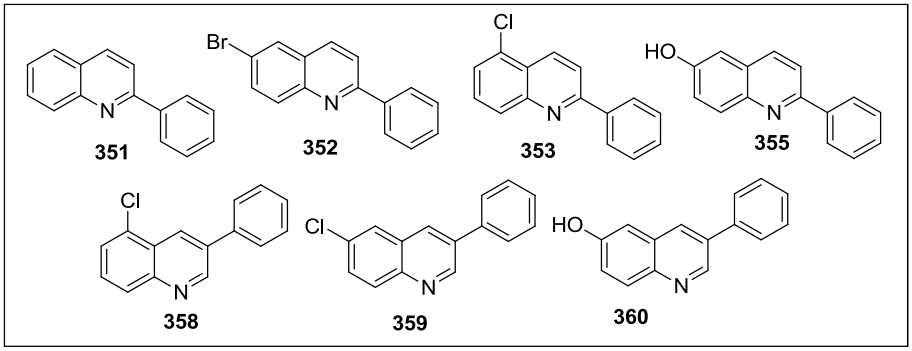
2.2.4 Conclusions

In summary, among the quinoline derivatives screened for their effect on brassinin detoxification, compounds **351**-**353**, **355** and **357**-**360** decreased the rate of brassinin metabolism (Figure **2.26**). Especially, cultures of compounds **352**, **353**, **357**, **358** and **360** co-incubated with brassinin (**17**) demonstrated $t_{1/2}$ and t_{Br} values greater than the respective values in control cultures. These results demonstrated a significant effect of compounds **352**, **353**, **357**, **358** and **360** in decreasing the rate of brassinin metabolism. Further studies indicated that compounds **351**-**353, 355** and **358**-**360** were likely metabolized in the cultures of *L. maculans* (virulent on canola) while compound **357** was not (Figure **2.26**). Among the isoquinoline derivatives, compounds **362**, **365** and **366** slowed down the rate of brassinin metabolism. Values of $t_{1/2}$ and t_{Br} in the cultures of compounds **362**, **365** and **366** co-incubated with brassinin (**17**) were greater than the respective values in control cultures. Further studies indicated that compounds **362**, **365** and **366** were not likely metabolized in the cultures of *L. maculans* (virulent on canola) (Figure **2.26**). The remaining compounds **350**, **354**, **356**, **361**, **363** and **364** did not show a detectable effect on the rate of brassinin metabolism in the cultures of *L. maculans* (virulent on canola) (Figure **2.27**). Additional experiments indicated that these compounds were likely metabolized in the cultures of *L. maculans* (virulent on canola).

Among the quinoline compounds **351**-**353**, **355** and **357**-**360** (Figure **2.26**) which delayed the metabolism of brassinin (**17**), compounds **352**, **358** and **359** displayed < 30% inhibition of the growth of *L. maculans* at 0.50 mM. However, all the three compounds **352**, **358** and **359** were likely metabolized by *L. maculans* (virulent on canola). Overall, compound **352** (0.10 mM) with a $t_{1/2}$ value of 65 h and only 27% inhibition of *L. maculans* growth at 0.50 mM appeared to be the most promising compound towards lead optimization. However, compound **352** seemed to be metabolized rapidly in the cultures of *L. maculans* (virulent on canola). Among the isoquinolines **362**, **365** and **366** (Figure **2.26**) which delayed the metabolism of brassinin (**17**), none displayed < 30% inhibition of the growth of *L. maculans* at 0.50 mM. On the other hand, compound **362** showed complete growth inhibition of *L. maculans* at 0.50 mM, and also a strong effect in decreasing the rate of brassinin (**17**)

metabolism ($t_{1/2}$ value in screening studies ~ 45 h). However, compound **362** was not likely metabolized in the fungal cultures of *L. maculans* (virulent on canola).

(**A**)



(**B**)

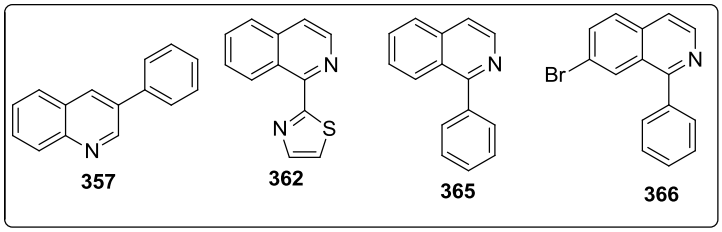


Figure 2.26 Compounds that slowed down the rate of brassinin metabolism in cultures of *Leptosphaeria maculans* (virulent on canola) in water: (A) that are likely metabolized **351**-**353**, **355** and **358**-**360**; (B) that are not likely metabolized **357**, **362**, **365** and **366**.

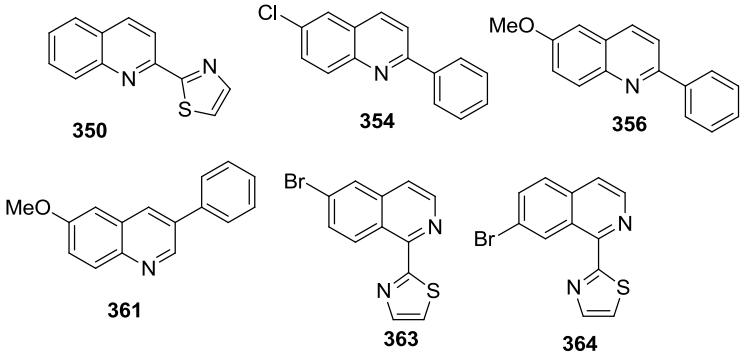


Figure 2.27 Compounds **350**, **354**, **356**, **361**, **363** and **364** that did not affect the rate of brassinin metabolism in cultures of *Leptosphaeria maculans* in water.

Considering the results from antifungal activities, screening for the effect on the decrease of brassinin detoxification rates and metabolic stability in cultures, compounds **352** and **362** seemed like promising structures. Thus, for a better understanding of the utility these compounds, several analogs of **352** and **362** have to be synthesized and tested for their antifungal activities, effect on brassinin detoxification and metabolic stability in fungal cultures.

In vitro evaluation of the compounds **352**, **362** and others for BOLm inhibition would be essential to further exploit the quinoline and isoquinoline based scaffolds in optimization studies. The summary of the results of screening, antifungal activities and possible metabolic stability of compounds **350**-**366** in fungal cultures tested towards evaluation of paldoxin-like properties is presented in table **2.6**.

Table 2.6 Concentration of brassinin (**17**) remaining in cultures of *Leptosphaeria maculans* incubated with compounds **350-366**.

Compound coincubated with brassinin (**17**)/ antifungal activity [c] (0.50 mM)	Recovery (%)[d] of brassinin (**17**)
Brassinin (**17**)/ 62 ± 4	50% (6 h); n.d. (48 h)
2-(Thiazol-2-yl)quinoline (**350**)[a]/ 54 ± 3	50% (6 h); 31% (24 h)
2-Phenylquinoline (**351**)[b]/ 54 ± 2	50% (18 h); 12% (48 h)
6-Bromo-2-phenylquinoline (**352**)[b]/ 27 ± 4	50% (65 h); 39% (72 h)
5-Chloro-2-phenylquinoline **(353)**[b]/ 39 ± 2	50% (24 h); 19% (48 h)
6-Chloro-2-phenylquinoline **(354)**[a]/ 26 ± 4	50% (6 h); 11% (24 h)
6-Hydroxy-2-phenylquinoline **(355)**[b]/ 68 ± 5	50% (15 h); 20% (48 h)
6-Methoxy-2-phenylquinoline **(356)**[b]/ 36 ± 4	50% (14 h); n.d. (48 h)
3-Phenylquinoline **(357)**[a]/ c.i.	50% (48 h); 29% (72 h)
5-Chloro-3-phenylquinoline **(358)**[b]/ ± 3	50% (24 h); 11% (48 h)
6-Chloro-3-phenylquinoline **(359)**[b]/ 28 ± 3	50% (6 h); 11% (24 h)
6-Hydroxy-3-phenylquinoline **(360)**[b]/ 73 ± 5	50% (19 h); 10% (48 h)
6-Methoxy-3-phenylquinoline **(361)**[b]/ c.i.	50% (14 h); n.d. (48 h)
1-(Thiazol-2-yl)isoquinoline (**362**)[a]/ c.i.	50% (45 h); 24 (72 h)
6-Bromo-1-(thiazol-2-yl)isoquinoline (**363**)[b]/ 75 ± 6	50% (9 h); n.d. (48 h)
7-Bromo-1-(thiazol-2-yl)isoquinoline (**364**)[b]/ 82 ± 5	50% (6 h); n.d. (48 h)
1-Phenylisoquinoline (**365**)[a]/ 54 ± 5	50% (24 h); 47% (48 h)
7-Bromo-1-phenylisoquinoline **(366)**[a]/ 47 ± 5	50% (26 h); 18% (48 h)

[a] not likely metabolized by *L. maculans*; [b] likely metabolized by *L. maculans*; [c] values were calculated using the formula: % inhibition = 100 – [(growth on amended/growth in control) × 100]; [d] molar percentages were determined using a calibration curve and are average of experiments conducted in triplicates ± standard deviation; c.i. = complete inhibition; n.d. = not detected.

Chapter 3: Conclusions and future work

It was established that rapalexin A (**22**) can resist transformation by *L. maculans*. Compound **22** is the third example of a phytoalexin, after spirobrassinin (**27**) and camalexin (**32**), that was not metabolized by the blackleg fungus. On the other hand, erucalexin (**29**) in contrast to its regioisomer 1-methoxyspirobrassinin (**28**), was found to be metabolized by *L. maculans* (virulent on canola) by reduction. Brassinin (**17**) and its regioisomer isobrassinin (**111**) were metabolized differently by *L. maculans* (BJ-125). During screening of different brassinin related structures, **111**, **153**, **320**, **325**, **327**, **331**, **333** and **342**, it was established that dithiocarbamates **111**, **325**, **327**, **331**, **342** were metabolized differently than brassinin (**17**). These results are in agreement with earlier findings (Pedras, Jha et al., 2007; Pedras, Minic et al., 2008) that compounds **96**, **111**, **153**, **320**, **325**, **327**, **331**, **333** and **342** were not substrates of BOLm. Detoxification of dithiocarbamates **111**, **325**, **327**, **331**, **342** and dithiocarbonates **153** and **333** by *L. maculans* occurred via a hydrolysis reaction. These broad range reactions demonstrated that the hydrolase(s) involved in these transformations are not very selective.

Replacement of indolyl and naphthylyl moieties in the first generation inhibitors of BOLm with quinoline and isoquinoline moieties provided a basis for the design of several potential inhibitors of brassinin detoxification by *L. maculans*. However, most of the substituted quinoline and isoquinoline derivatives **353-361** and **366** incorporating phenyl substitution at the 2 or 3 positions showed less effects in decreasing the rate of brassinin detoxification relative to compounds **351**, **357** and **362**. There was no SAR observed among the compounds in regards to the effects such as decreasing the rate of brassinin detoxification and antifungal activity. Overall, compounds **352** and **362** showed substantial effects in decreasing the rate of brassinin detoxification. However, compound **352** but not **362** had low antifungal activity against *L. maculans*. This demonstrated the scope for further investigation of compounds based on the structures **352**, **362** and others as inhibitors of brassinin detoxification. Further studies are required to understand the metabolic stability in *L. maculans* (virulent on canola) and

BOLm inhibition activities of compounds **350**-**366**. In addition, compounds derived from other potential heterocyclic skeletons need to be explored to probe for their effect in decreasing the rate of brassinin detoxification, BOLm inhibition activity and antifungal activity against *L. maculans* (virulent on canola).

Future work

- Test compounds **350**-**366** for inhibition of brassinin transformation using purified BOLm.
- Determine the metabolic stability of compounds **350**-**366** in cultures of *L. maculans* (virulent on canola).

Chapter 4: Experimental

4.1 General methods

All reagents and chemicals were purchased either from Aldrich or Alfa Aesar. Solvents used in syntheses were dried prior to use according to established procedures (pyridine and DMF with 3 Å molecular sieves, THF and Et_2O over sodium and benzophenone, DCM, CH_3CN, and benzene over CaH_2) (Leonard, Lygo et al., 1995).

Analytical thin layer chromatography (TLC) was carried out on alumina sheets (Merck, 60 F_{254} 5 × 2 cm × 0.2 mm) pre-coated with silica gel. Compounds developed on the TLC plates were visualized under UV light (254/366 nm) and/or by dipping in a solution of 5% (w/v) aqueous phosphomolybdic acid containing 1% (w/v) ceric sulfate and 4% (v/v) H_2SO_4, followed by charring on a hot plate.

Flash column chromatography (FCC) was carried out using silica gel grade 60, mesh size 230 – 400 Å. Preparative thin layer chromatography (prep TLC) was carried out on silica gel plates, Kieselgel 60 F_{254} (20 × 20 cm × 0.25 mm).

NMR spectra were obtained on Bruker Avance 500 spectrometers. For ^1H NMR (500 MHz), the chemical shift values (δ) are reported in parts per million (ppm) relative to tetramethylsilane. The δ values were referenced to $CDCl_3$ ($CHCl_3$ at 7.27 ppm), and CD_3CN (CHD_2CN at 1.94 ppm). First-order behavior was assumed in analysis of ^1H NMR spectra and multiplicities are indicated by the following notations; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br = broad. Spin coupling constants (*J* values) are reported to the nearest 0.5 Hz. ^{13}C data were collected on the Bruker Avance 500 spectrometers at 125.8 MHz. The ^{13}C chemical shift (δ values) were referenced to $CDCl_3$ (77.2 ppm) and CD_3CN (118.7 ppm).

HPLC analysis was carried out with Agilent high performance liquid chromatography instruments equipped with quaternary pump, automatic injector, and diode array detector (DAD, wavelength range 190–600 nm), degasser, and a column, having an in-line filter. Elution methods: method A: column Eclipse XDB-C18 (5 μm particle size silica, 4.6 i.d. × 150 mm), mobile phase H_2O–MeOH (1:1, v/v) to MeOH, for 25.0 min, linear gradient, and at a flow rate 0.75 ml/min; method B (for amines both solvents

contain 0.01% propanamine): column Zorbax ODS (3.5 μm particle size silica, 3.0 i.d. × 100 mm), mobile phase H_2O–MeOH (4:6, v/v) to MeOH, for 10.0 min, linear gradient, at a flow rate of 0.50 ml/min; method C: column Zorbax ODS (3.5 μm particle size silica, 3.0 i.d. × 100 mm), mobile phase H_2O–ACN (3:1, v/v) to ACN, for 40.0 min, linear gradient, at a flow rate of 0.40 ml/min. HPLC–DAD-ESI-MS analysis was carried out with an Agilent 1100 series HPLC system equipped with an autosampler, binary pump, degasser, and a diode array detector connected directly to a mass detector (Agilent G2440A MSD-Trap-XCT ion trap mass spectrometer) with an electrospray ionization (ESI) source.

Fourier transform infrared (FTIR) spectra were recorded on Bio-Rad FTS-40 spectrometers. Spectra were measured by the diffuse reflectance method on samples dispersed in KBr. High resolution mass spectra (HRMS-EI) were obtained on a VG 70 SE mass spectrometer.

Minimal medium (MM) contains the following chemicals:

Solution 1: KNO_3 (3.12 g/l, 30.8 mM), K_2HPO_4 (0.75 g/l, 4.31 mM), KH_2PO_4 (0.75 g/l, 5.51 mM), NaCl (0.10 g/l, 1.71 mM), asparagine (0.28 g/l, 2.12 mM);

Solution 2: $CaCl_2 \cdot 2H_2O$ (0.10 g/l, 0.68 mM), $MgSO_4 \cdot 7H_2O$ (0.50 g/l, 2.03 mM);

Solution 3: $ZnSO_4 \cdot 7H_2O$ (0.395 mg/l, 1.37 μM), $CuSO_4 \cdot 5H_2O$ (0.079 mg/l, 0.32 μM), $MnSO_4 \cdot 4H_2O$ (0.0405 mg/l, 0.18 μM), MoO_3 (85%, 0.05 mg/l, 0.12 μM), ferric citrate (0.535 mg/l, 2.16 μM), $Na_2B_4O_7 \cdot 10H_2O$ (0.0375 mg/l, 0.10 μM);

Solution 4: thiamine (0.1 mg/l, 0.38 μM);

Glucose: (15 g/l, 83.3 mM).

Solutions 1 and 3 and glucose were mixed and the pH of the mixed solution was adjusted to 6.55 and autoclaved. The autoclaved solution was combined with solutions 2 and 4 (solutions 2 and 4 were sterilized, separately). Canadian virulent isolates of *L. maculans* (BJ 125) were grown in Erlenmeyer flasks (125 ml) containing MM (50 ml) inoculated with fungal spores (5×10^7/50 ml) and incubated on a shaker at 120 rpm, at 23 ± 2 °C (Pedras and Biesenthal, 2000). Cultures were grown in triplicate.

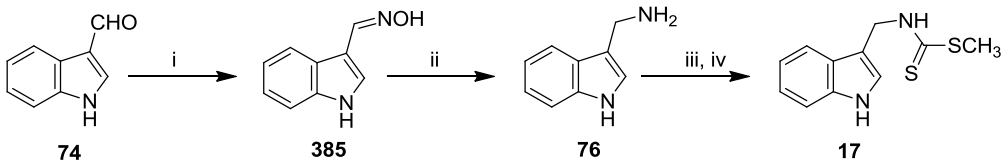
4.2 Fungal isolates

Fungal isolates were provided by Agriculture and Agri-Food Canada Research Station, Saskatoon and University of Alberta, Edmonton. For spore collection, virulent isolates BJ-125 and UAMH 9410 were subcultured on V8 agar under continuous light, at 24 ± 2 °C for 14 days. Spores were collected by centrifugation of the spore suspension (Pedras and Jha, 2006) diluted, counted under a microscope using haemocytometer and stored at -20 °C.

4.3 Phytoalexins and analogs

4.3.1 Synthesis of phytoalexins

4.3.1.1 Brassinin (17)



Reagents and conditions: (i) $NH_2OH.HCl$, Na_2CO_3, 95%; (ii) $NiCl_2.6H_2O$, $NaBH_4$, MeOH, 70%; (iii) Et_3N, pyridine, CS_2, 0 °C; (iv) MeI, 5 °C, 73% (Takasugi, Monde et al., 1988).

Compound **17** was prepared according to a reported procedure. A solution of $NH_2OH.HCl$ (957 mg, 13.8 mmol) and Na_2CO_3 (803 mg, 7.58 mmol) in water (10 ml) was added to a solution of indolyl-3-carboxaldehyde (**74**, 1.0 g, 6.9 mmol) in EtOH (25 ml) and stirred for 1 h at 80 °C. The reaction mixture was concentrated using rotary evaporator and the resulting precipitate was filtered to afford indolyl-3-carboxaldoxime (**385**, 1050 mg, 6.56 mmol) as an off-white solid in 95% yield (Pedras, Borgmann et al., 1992).

$NaBH_4$ (1075 mg, 28.44 mmol) was added to a solution of oximes **385** (700 mg, 4.37 mmol) and $NiCl_2.6H_2O$ (1039 mg, 4.37 mmol) in MeOH (10 ml) and stirred at 0 °C for 10 min. The reaction mixture was then diluted with 2:1 mixture of water and aq. NH_3 (50 ml), filtered and extracted with EtOAc (3 x 100 ml). The combined organic

extracts were dried over Na_2SO_4 and concentrated. The residue was subjected to FCC ($CHCl_3$/MeOH/aq.NH_3, 80:20:1) to afford 3-indolylmethanamine (**76**, 450 mg, 3.08 mmol) in 70% yield as white solid (Kutschy, Dzurilla et al., 1998).

CS_2 (199 μl, 3.31 mmol) was added to a solution of amine **76** (440 mg, 3.01 mmol) and Et_3N (837 μl, 6.02 mmol) in pyridine (3 ml) cooled to 0˚ C, and stirred at same temperature for 10 min. Next, CH_3I (282 μl, 4.51 mmol) was added and the reaction mixture was stirred at r.t. for 30 min. The reaction mixture was poured into 1.5 M H_2SO_4 (50 ml) and extracted with EtOAc (2 x 100 ml). The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was subjected to FCC on silica gel (20% EtOAc/Hex) to afford brassinin (**17**, 521 mg, 2.20 mmol) as an off-white solid in 73% yield (Pedras, Borgmann et al., 1992).

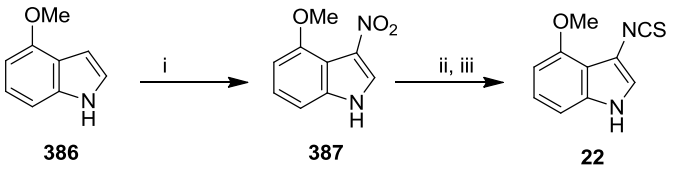
HPLC t_R = 11.9 min (method A).

^{1}H NMR (500 MHz, CD_3CN) δ 9.25 (br s, 1H, D_2O exchangeable), 8.24 (br s, 1H, D_2O exchangeable), 7.63 (d, *J* = 8 Hz, 1H), 7.43 (d, *J* = 8 Hz, 1H), 7.31 (d, *J* = 2 Hz, 1H), 7.16 (dd, *J* = 8, 8 Hz, 1H), 7.08 (dd, *J* = 8, 8 Hz, 1H), 5.04 (d, *J* = 5 Hz, 2H), 2.55 (s, 3H) and minor signals 4.77 (d) and 2.64 (s) (ca. 1/10 itensity of the major peaks) due to a rotamer.

^{13}C NMR (125 MHz, CD_3CN) δ 199.2, 137.8, 128.2, 126.2, 123.2, 120.7, 120.0, 112.9, 111.9, 43.6, 18.6.

HRMS-EI *m/z*: measured 236.0438 ($[M]^+$, calcd. 236.0442 for $C_{11}H_{12}N_2S_2$). MS (EI) *m/z* (% relative int.): 236 (34), 162 (12), 130 (100), 129 (32), 102 (16).

4.3.1.2 Rapalexin A (22)



Reagents and conditions: (i) $AgNO_3$, benzoyl chloride, CH_3CN, 21%; (ii) Pd/C, AcOH, H_2; (iii) $CSCl_2$, DCM, 19% over 2 steps (Pedras, Zheng et al., 2007d).

Compound **22** was prepared according to the reported procedure by Pedras et al (Pedras, Zheng et al., 2007d).

Benzoyl chloride (116 µl, 0.84 mmol) was added dropwise to a stirred solution of silver nitrate (137 mg, 0.81 mmol) in acetonitrile (1 ml) at 0 °C and further stirred for 10 min. This solution was added to a solution of 4-methoxyindole (**386**, 98 mg, 0.67 mmol) in acetonitrile (8 ml) at -20 °C with vigorous stirring. After 1 hr, the reaction mixture was brought to room temperature and stirred for additional 2 hrs. The reaction mixture was diluted with water (20 ml) and extracted with EtOAc (3×20 ml). The combined organic extract was washed with sodium carbonate (10% aqueous solution, 10 ml); dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by FCC on silica gel (hexane- EtOAc, 10:1) to afford 4-methoxy-3-nitroindole (**387,** 27 mg, 0.14 mmol) as a yellow powder in 21 % yield.

Platinum (IV) oxide (40 mg) was added to a solution of 4-methoxy-3-nitroindole (**387**, 75 mg, 0.39 mmol) in 95% EtOH (5 ml) and the mixture was hydrogenated at balloon pressure with vigorous stirring. After complete consumption of the starting material according to TLC, the reaction mixture was concentrated to 1/3 volume using a rotary evaporator, and immediately used in the next step. The reaction mixture in EtOH (1 ml) was added dropwise to a stirred mixture of DCM (3 ml) and thiophosgene (38 µl, 0.41 mmol) at room temperature. After stirring for 10 min, the mixture was diluted with water (10 ml) and extracted with DCM. (3×10 ml). The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was subjected to FCC on silica gel (20% EtOAc/Hex) to afford rapalexin A (**22**, 15 mg, 0.07 mmol) in 19% yield as a white solid (Pedras, Zheng et al., 2007d).

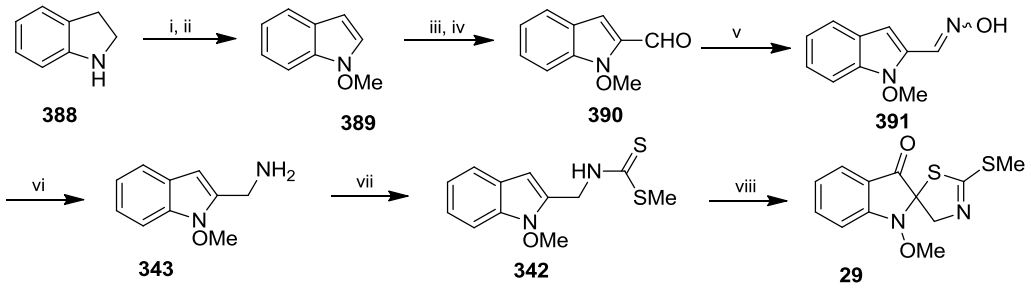
HPLC t_R = 16.0 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.97 (brs, NH), 7.17 (t, *J* = 8 Hz, 1H), 7.08 (d, *J* = 2 Hz, 1H), 6.95 (d, *J* = 8 Hz, 1H), 6.58 (d, *J* = 8 Hz, 1H), 4.00 (s, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 154.1, 135.9, 136.1, 125.1, 118.3, 114.2, 108.1, 104.9, 101.1, 55.5.

HRMS-EI *m/z*: measured 204.0356 ([M] $^+$, calcd. 204.0357 for $C_{10}H_8N_2OS$). MS (EI) *m/z* (% relative int.): 204 [M^+] (100), 189 (57), 173 (11), 161 (30), 149 (13), 101 (22).

4.3.1.3 ***Erucalexin (29)***



Reagents and conditions: (i) $Na_2WO_4.2H_2O$, 30% H_2O_2; (ii) Me_2SO_4, K_2CO_3, 55% (Kawasaki and Kodama et al., 1991); (iii) *t*-BuLi, THF, -78 °C; (iv) DMF, 86%; (v) $NH_2OH.HCl$, Na_2CO_3, $EtOH/H_2O$, 93%; (vi) $NaBH_3(CN)$, $TiCl_3$, NH_4OAc, MeOH; (vii) Pyr, Et_3N, CS_2, CH_3I, 51%; (V) CrO_3, $AcOH/H_2O$, 32% (Pedras, Suchy et al., 2006).

Compound **29** was prepared according to a procedure by Pedras et al (Pedras, Suchy et al., 2006). A solution of $Na_2WO_4.2H_2O$ (485 mg, 1.47 mmol) in water (5 ml) was added to the stirred solution of indoline (**388**, 1000 mg, 8.4 mmol) in MeOH (40 ml) and the reaction mixture was cooled to -15 ºC and stirred for 10 min. Next, a solution of 30% H_2O_2 (10 ml, 72.6 mmol) in MeOH (15 ml) was added slowly to the reaction mixture and stirring was continued at -15 ºC. After 30 minutes, solid K_2CO_3 (9.28 g, 67.2 mmol) and dimethyl sulphate (2.1 ml, 22.26 mmol) were added with vigorous stirring and the reaction mixture was stirred for further 1.5 h at 8-13 ºC. The reaction mixture was then poured into water (120 ml) and extracted with Et_2O (2×100 ml). The combined organic extract was dried over Na_2SO_4 and concentrated under reduced

pressure. The residue was subjected to FCC on silica gel (20% DCM/Hex) to afford 1-methoxyindole (**389**, 674 mg, 4.58 mmol) in 55% yield (Kawasaki, Kodama et al., 1991).

t-BuLi in pentane (4.3 ml, 4.3 mmol) was added dropwise to a solution of 1-methoxyindole (**389**, 420 mg, 2.86 mmol) in dry THF (10 ml, under an Ar atmosphere), cooled to −78 °C, and stirred at same temperature. After 15 min, DMF (0.75 ml, 4.8 mmol) was added and the reaction temperature was gradually raised to r.t under continued stirring. After 1 hr, the reaction mixture was cooled to 0 °C, diluted with 1 M HCl (30 ml) and extracted with Et_2O. The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was subjected to FCC on silica gel (20% EtOAc/Hex) to afford 1-methoxyindolyl-2-carboxaldehyde (**390,** 435 mg, 2.48 mmol, 86%) as an off-white solid.

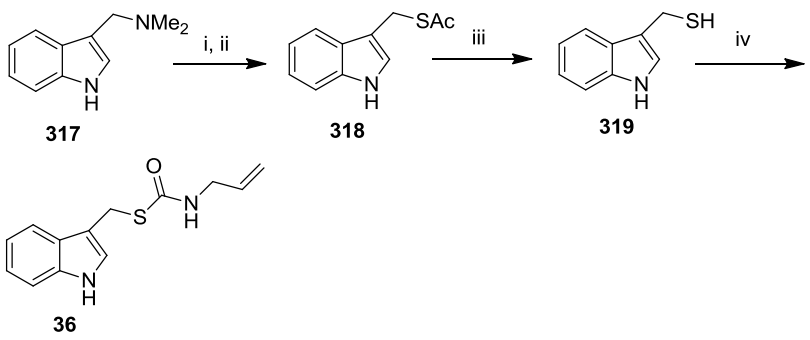
A solution of $NH_2OH{\cdot}HCl$ (312 mg, 4.52 mmol) and Na_2CO_3 (209 mg, 1.98 mmol) in water (3 ml) was added to 1-methoxyindolyl-2-carboxaldehyde (**390,** 500 mg, 2.82 mmol) in EtOH (10 ml) and stirred at room temperature. After 20 min, the reaction mixture was diluted with brine (30 ml) and extracted in EtOAc. The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure to yield pure 1-methoxyindolyl-2-carboxaldoxime (**391**, 497 mg, 93%). $AcONH_4$ (891 mg, 11.57 mmol) and $NaBH_3CN$ (663 mg, 10.53 mmol) were added to a cooled solution of 1-methoxyindolyl-2-carboxaldoxime (**391**, 200 mg, 1.05 mmol) in MeOH (10 ml) and stirred at 0 °C. After 5 min, a solution of $TiCl_3$ [prepared by mixing commercially available 30% $TiCl_3$ in 2 M HCl (4.9 ml) with 1 M NaOH solution (3.2 ml)] was added and the mixture was stirred for 20 min at 0 °C. The reaction mixture was then diluted with 2:1 mixture of H_2O and NH_4OH (50 ml) and extracted with EtOAc. The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure to yield the crude 1-methoxyindolyl-2-methanamine (**343**), which was taken to next step without purification. CS_2 (75 µl, 1.26 mmol) was added to a cooled solution of this amine **343** (1.05 mmol) and Et_3N (219 µl, 1.58 mmol) in pyridine (2 ml) and stirred at 0 °C for 30 min. Next, CH_3I (99 µl, 1.58 mmol) was added and the mixture was stirred at same temperature. After 1 h, the reaction mixture was diluted with 1.5 M H_2SO_4 (20

ml) and extracted with Et_2O. The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by FCC on silica gel (20% EtOAc/Hex) to afford 1-methoxyisobrassinin (**342**, 142 mg, 0.53 mmol) as colorless oil in 51% yield (Pedras, Suchy et al., 2006).

A solution of CrO_3 (244 mg, 2.43 mmol) in water (0.75 ml) was added to a solution of 1-methoxyisobrassinin (**342**, 118 mg, 0.44 mmol) in acetic acid (2.5 ml) and stirred at room temperature. After 5 min, the reaction mixture was diluted with brine (20 ml) and was extracted using EtOAc. The combined organic extract was washed with 10% K_2CO_3 solution (20 ml), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by FCC (SiO_2; hexane–acetone, 5:1) to afford erucalexin (**29**, 38 mg, 0.13 mmol) in 32% yield as a brown solid (Pedras, Suchy et al., 2006).

HPLC t_R = 14.9 min (method A).
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.68 (d, *J* = 8 Hz, 1H), 7.63 (ddd, *J* = 8, 8, 1 Hz, 1H), 7.22 (d, *J* = 8 Hz, 1H), 7.12 (dd, *J* = 8, 8 Hz, 1H), 4.73 (d, *J* = 16 Hz, 1H), 4.44 (d, *J* = 16 Hz, 1H), 3.97 (s, 3H), 2.61 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): δ 192.0, 165.8, 159.9, 138.0, 124.5, 123.7, 121.1, 114.0, 94.2, 71.8, 65.3, 15.7.
HRMS-EI *m/z*: measured 280.0337 ([M] $^+$, calcd. 280.0340 for $C_{12}H_{12}N_2O_2S_2$). MS (EI) *m*/*z* (% relative int.): 280 [M^+] (46), 249 (81), 207 (32), 176 (100), 132 967), 105 (76), 87 (36).

4.3.1.4 Brussalexin (36)



Reagents and conditions: (i) Me_2SO_4, H_2O; (ii) CH_3COSH, KOH, 70 °C, 66%; (iii) $NH_2NH_2.H_2O$, THF, 80 °C, 84%; (iv) Et_3N, allyl isocyanate, DCM, 86%.

Compound **36** was prepared by modification of the procedure by Pedras et al (Pedras, Zheng et al., 2007f). Dimethyl sulphate (435 µl, 4.59 mmol) was added dropwise to a suspension of grammine (**317**, 400 mg, 2.3 mmol) in water (5 ml) and stirred till the reaction medium turned homogeneous. Next, a solution of potassium thioacetate (5.05 mmol) [generated by dissolving thiolacetic acid (360 µl) and KOH (283 mg) in water (3 ml)] was added and the reaction mixture was heated to 70 °C under stirring. After 3 h, the reaction mixture was cooled to r.t. and extracted with Et_2O (3 × 50 ml). The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by FCC (SiO_2; 20% EtOAc/Hex) to afford indolyl-3-methylthioacetate (**318,** 309 mg, 1.50 mmol) as a pale brown oil in 66% yield (Benghait and Crooks, 1983).

Hydrazine hydrate (109 mg, 2.19 mmol) was added to a solution of indolyl-3-methylthioacetate (**318,** 150 mg, 0.73 mmol) in THF (4 ml) and refluxed for 45 min. The reaction mixture was concentrated, adsorbed on silicagel, and fractionated (5% EtOAc/Hex) to afford indolyl-3-methanethiol (**319**, 100 mg, 0.61 mmol) in 84% yield.
HPLC t_R = 11.2 min (method A).
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.89 (br s, NH), 7.68 (d, *J* = 8 Hz, 1H), 7.30 (d, *J* = 8 Hz, 1H), 7.20 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.14 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.05 (s, 1H), 3.95 (d, *J* = 7 Hz, 2H), 1.82 (t, *J* = 7 Hz, SH).

^{13}C NMR (125 MHz, $CDCl_3$): δ 136.5, 126.3, 122.6, 122.2, 119.8, 119.1, 115.8, 111.5, 19.8.

FTIR (KBr) ν_{max} cm^{-1}: 3410, 3054, 2922, 2557, 1618, 1550, 1456, 1350, 1223, 1093, 742.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 220, 276.

HRMS-EI *m/z*: measured 163.0453 ([M] $^+$, calcd. 163.0456 for C_9H_9NS). MS (EI) *m/z* (% relative int.): 163 [M^+] (20), 130 (100).

Allyl isocyanate (28 mg, 0.34 mmol) was added to a solution of indolyl-3-methanethiol (**319,** 50 mg, 0.31 mmol) and triethylamine (17 μl, 0.62 mmol) in DCM (2 ml) and stirred at r.t., for 20 min. After complete conversion of the starting material, the reaction mixture was concentrated and loaded on the column of silica gel and eluted with EtOAc-Hex (20:80, v/v) to afford brussalexin (**319,** 65 mg, 0.26 mmol) in 86% yield.

HPLC t_R = 10.4 min (method A).

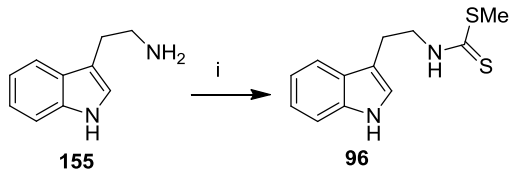
^{1}H NMR (500 MHz, $CDCl_3$): δ 8.14 (br s, 1H), 7.68 (d, *J* = 8 Hz, 1H), 7.35 (d, *J* = 8 Hz, 1H), 7.22 (ddd, *J* = 8, 8, 1 Hz, 1H), 7.18-7.15 (m, 1H), 5.82 (m, 1H), 5.45 (br s, 1H), 5.20 (d, *J* = 17 Hz, 1H), 5.15 (dd, *J* = 10, 1 Hz, 1H), 4.42 (s, 2H), 3.91 (br s, 2H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 167.5, 136.5, 133.9, 126.8, 123.7, 122.5, 119.9, 119.1, 117.0, 112.3, 111.5, 43.8, 25.7.

HRMS-EI *m/z*: measured 246.0832 ([M] $^+$, calcd. 246.0827 for $C_{13}H_{14}N_2OS$). MS (EI) *m/z* (% relative int.): 246 [M^+] (8), 163 (6), 130 (100), 102 (7).

4.3.2 Synthesis of phytoalexin analogs

4.3.2.1 ***Methyl tryptamine dithiocarbamate (96)***



Reagents and conditions: (i) Et_3N, Pyr, CS_2; CH_3I, 87%.

Compound **96** was synthesized as previously reported (Pedras and Okanga, 2000). A solution of tryptamine (**155,** 300 mg, 1.87 mmol) in THF (2 ml) was added to a suspension of sodium hydride (54 mg, 2.25 mmol) in THF (3 ml) and stirred at r.t for 5 min. Next, CS_2 (135 µl, 2.246 mmol) followed by MeI (5 µl, 2.81 mmol) were added and the reaction mixture was stirred at r.t. After 30 min, the reaction mixture was diluted with ice cold water (30 ml) and extracted with EtOAc (2 × 30 ml). The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure to yield a residue which was separated by FCC (SiO_2; 20% EtOAc/Hex) to afford methyl tryptaminedithiocarbamate (**96**, 390 mg, 1.47 mmol) as a yellow solid in 83% yield (Pedras and Okanga, 2000).

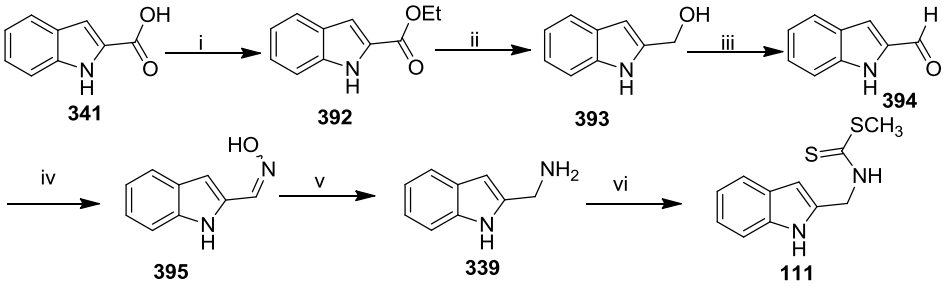
HPLC t_R = 24.6 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.10 (br s, NH), 7.64 (d, *J* = 8 Hz, 1H), 7.39 (d, *J* = 8 Hz, 1H), 7.25 (dd, *J* = 8, 8 Hz 1H), 7.17 (dd, *J* = 8, 8 Hz 1H), 7.05 (s, 1H), 7.02 (br s, NH), 4.10-4.07 (m, 2H), 3.16-3.10 (m, 2H), 2.60 (s, 3H) and minor signals (approx. 1/3rd the intensity of major ones) due to rotamers at 7.60, 3.77, 2.70.

^{13}C NMR (125 MHz, $CDCl_3$): δ 199.0, 136.6, 127.4, 122.6, 122.4, 119.9, 118.9, 112.5, 111.5, 47.4, 24.8, 18.3 and minor signals (approx. 1/3rd the intensity of major ones) due to rotamers at 118.7, 46.3, 24.8, 19.1.

HRMS-EI *m/z*: measured 250.0593 ([M] $^+$, calcd. 250.0598 for $C_{12}H_{14}N_2S_2$). MS (EI) *m/z* (% relative int.): 250 [M^+] (6), 202 (16), 143 (63), 130 (100), 115 (7), 103 (6), 77 (9).

4.3.2.2 ***Isobrassinin (111)***



Reagents and conditions: (i) EtOH, H_2SO_4, 115 °C, 87%; (ii) $LiAlH_4$, THF, 0 °C; (iii) MnO_2, DCM, 80%; (iv) $NH_2OH.HCl$, Na_2CO_3, $EtOH/H_2O$, 94%; (v) $NaBH_4$, $NiCl_2.6H_2O$, MeOH; (vi) Pyr, Et_3N, CS_2, CH_3I, 44% (Pedras, Suchy et al., 2006).

Isobrassinin (**111**) was synthesized as previously reported (Pedras, Suchy et al., 2006). H_2SO_4 (4 drops) was added to a solution of indolyl-2-carboxylic acid (**341**, 900 mg, 5.59 mmol) in EtOH (15 ml) and refluxed for 20 hours. The reaction mixture was then cooled to room temperature, diluted with water (20 ml) and extracted with DCM (30 ml). The combined organic extract was washed with 10% Na_2CO_3 (2 × 20 ml) followed by water (30 ml); dried over Na_2SO_4 and concentrated under reduced pressure to afford ethyl indolyl-2-carboxylate (**392**, 920 mg, 4.87 mmol) in 87% yield.

LAH (174 mg, 4.6 mmol) was added in small portions to a cooled solution of carboxylate **392** (720 mg, 3.83 mmol) in dry THF (17 ml) at 0 °C and stirred at r.t. After 1 h, the reaction was quenched with 5 N NaOH (1 ml) and the precipitate was filtered over celite. The filtrate was dried over Na_2SO_4 and concentrated under reduced pressure to afford indolyl-2-methanol (**393**, 546 mg). MnO_2 (1.9 g, 22.24 mmol) was added to the crude alcohol **393** (545 mg, 3.71 mmol) dissolved in DCM (20 ml) and the mixture was stirred at room temperature. After 18 h, MnO_2 was filtered off, and the filtrate was concentrated under reduced pressure to yield indole-2-carboxaldehyde (**394**, 438 mg, 3.02 mmol) as an off-white solid in 80% yield (Meyer and Kruse, 1984).

A solution of $NH_2OH.HCl$ (624 mg, 8.98 mmol) and Na_2CO_3 (595 mg, 5.61 mmol) in water (5 ml) was added to a solution of indole-2-carboxaldehyde (**394**, 438 mg, 2.24 mmol) in EtOH (10 ml) and the reaction mixture was refluxed at 95 °C. After 1 h,

EtOH was concentrated under reduced pressure, and the residue was diluted with water (5 ml). The resulting precipitate was vacuum filtered to afford E/Z- indole-2-carboxaldoximes (**395**, 452 mg, 2.82 mmol) in 94% yield.

$NaBH_4$ (691 mg, 18.26 mmol) was added to a solution of oximes **395** (450 mg, 2.81 mmol) and $NiCl_2.6H_2O$ (668 mg, 2.81 mmol) in MeOH (20 ml) and the mixture was stirred at r.t. After 10 min, the reaction mixture was diluted with 2:1 mixture of H_2O - aqueous NH_4OH (35 ml), filtered and extracted with EtOAc (3 x 100 ml). The combined organic extract was dried over Na_2SO_4 and concentrated to afford crude indolyl-2-methanamine **339**. CS_2 (331 µl, 5.50 mmol) was added to a solution of crude amine (**339**, 402 mg, 2.75 mmol) and Et_3N (142 µl, 5.50 mmol) in DCM (1.5 ml), cooled to 0 °C, and stirred at same temperature. After 30 min, CH_3I (225 µl, 3.58 mmol) was added and the reaction mixture was stirred at r.t., for 1 h. The reaction mixture was poured into 1.5 M H_2SO_4 (50 ml) and extracted with EtOAc (2 x 100 ml). The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was subjected to FCC on silica gel (20% EtOAc/Hex) to afford isobrassinin (**111**, 282 mg, 1.19 mmol) as a pale brown solid in 44% yield (Pedras, Suchy et al., 2006).

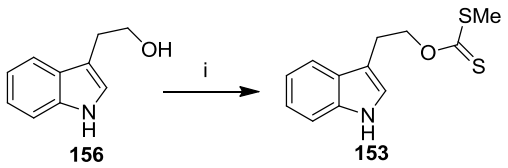
HPLC t_R = 14.0 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.91 (br s, NH), 7.59 (d, *J* = 8 Hz, 1H), 7.35 (d, *J* = 8 Hz, 2H), 7.21 (dd, *J* = 7.5, 8 Hz, 1H), 7.12 (dd, *J* = 7.5, 8 Hz, 1H), 6.42 (s, 1H), 5.05 (d, *J* = 5.5 Hz, 2H), 2.68 (s, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 201.3, 136.4, 134.6, 127.6, 122.7, 120.7, 120.2, 111.4, 102.2, 43.5, 18.7.

HRMS-EI *m/z*: measured 236.0450 ($[M]^+$, calcd. 236.0442 for $C_{11}H_{12}N_2S_2$). MS (EI) *m/z* (% relative int.): 236 [M^+] (28), 188 (17), 163 (13), 130 (100), 103 (7), 69 (15).

4.3.2.3 Methyl tryptophol dithiocarbonate (153)



Reagents and conditions: (i) NaH, THF, CS_2; CH_3I, 90% (Pedras and Jha, 2006).

A solution of tryptophol (**156,** 50 mg, 0.311 mmol) in THF (2 ml) was added to a suspension of sodium hydride (15 mg, 0.373 mmol) in THF (1 ml) and stirred at r.t. After 5 min, CS_2 (22 µl, 0.373 mmol) followed by MeI (40 µl, 0.622 mmol) were added and the reaction mixture was stirred at r.t. After 20 min, the reaction mixture was diluted with ice-cold water and extracted with EtOAc. The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure to yield a residue which was purified by FCC (SiO2; 20% EtOAc/Hex) to afford methyl tryptophol dithiocarbonate (**153**, 64 mg, 82%, 0.26 mmol) as a white solid.

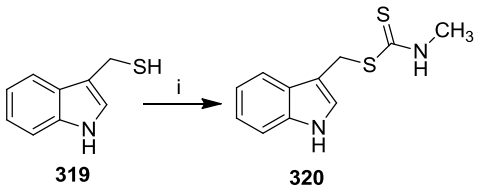
HPLC t_R = 20.1 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.02 (br s, NH), 7.68 (d, *J* = 8 Hz, 1H), 7.39 (d, *J* = 8 Hz, 1H), 7.25 (dd, *J* = 8, 8 Hz 1H), 7.17 (dd, *J* = 8, 8 Hz 1H), 7.08 (s, 1H), 4.90 (t, *J* = 7 Hz, 2H), 3.31 (t, *J* = 7 Hz, 2H), 2.57 (s, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 216.1, 136.4, 127.5, 122.5, 122.4, 119.7, 119.0, 111.7, 111.4, 73.9, 24.5, 19.1.

HRMS-EI *m/z*: measured 251.0434 ($[M]^+$, calcd. 251.0439 for $C_{12}H_{13}NOS_2$). MS (EI) *m/z* (% relative int.): 251 [M^+] (3), 143 (100), 130 (14), 115 (11).

4.3.2.4 Methyl indolyl-3-methylcarbamodithioate (320)



Reagents and conditions: (i) Et_3N, MeNCS, DCM, 76%.

Methyl isothiocyanate (49 mg, 0.67 mmol) was added to a solution of indolyl-3-methanethiol (**319,** 100 mg, 0.613 mmol) and triethylamine (200 µl, 1.47 mmol) in DCM (3 ml) and stirred at r.t. for 20 min. After complete conversion of the starting material, the reaction mixture was concentrated, loaded on the column of silica gel and eluted with EtOAc-Hex (20:80, v/v) to afford the product **320 (**110 mg, 0.46 mmol) in 76% yield.

HPLC t_R = 11.2 (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.20 (br s, NH), 8.01 (br s, NH), 7.63 (d, *J* = 8 Hz, 1H), 7.41 (d, *J* = 8 Hz, 2H), 7.29 (s, *J* = 2 Hz, 1H), 7.17 (dd, *J* = 8, 8 Hz, 1H), 7.08 (dd, *J* = 7, 7 Hz, 1H), 4.69 (s, 2H), 3.12 (s, 3H), minor rotamer peaks were found at δ 4.78, and 2.93 (ca. 1.7 : 0.3).

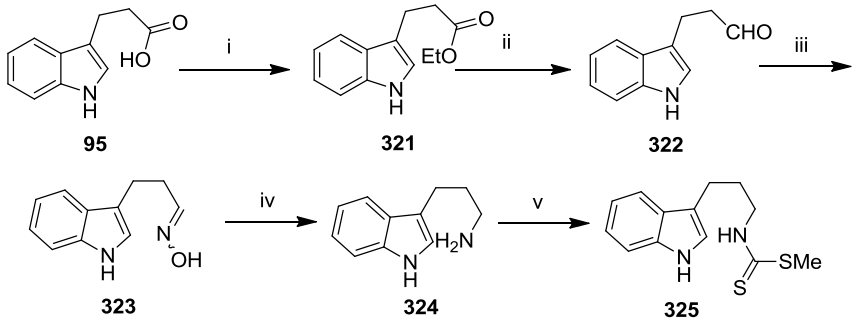
^{13}C NMR (125 MHz, $CDCl_3$): δ 199.4, 137.8, 128.0, 125.7, 123.3, 120.6, 120.0, 112.9, 110.8, 34.3, 32.1.

FTIR (KBr) ν_{max} cm^{-1}: 3403, 3323, 2928, 1509, 1455, 1339, 1031, 940, 744.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 219, 271.

HRMS-EI *m/z*: measured 236.0435 ([M] $^+$, calcd. 236.0442 for $C_{11}H_{12}N_2S_2$). MS (EI) *m/z* (% relative int.): 236 [M^+] (1), 163 (19), 130 (100), 103, (7), 76 (18).

4.3.2.5 Methyl 3-(3-indolyl)propyldithiocarbamate (325)



Reagents and conditions: (i) EtOH, H_2SO_4, 115°C, 96%; (ii) DiBAl-H, toluene, -78 °C, 90%; (iii) $NH_2OH.HCl$, Na_2CO_3, EtOH/H_2O, 97%; (iv) $NaBH_4$, $NiCl_2.6H_2O$, MeOH; (v) Pyr, Et_3N, CS_2, CH_3I, 43% (Gaspari, Banerjee et al., 2006).

H_2SO_4 (4 drops) was added to a solution of indolyl-3-propanoic acid (**95**, 500 mg, 2.67 mmol) in EtOH (15 ml) and the reaction mixture was refluxed for 20 h. The reaction mixture was then cooled to room temperature; diluted with water (20 ml) and extracted in DCM (30 ml). The combined organic extract was washed with 10% Na_2CO_3 (2 × 20 ml) followed by water, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was subjected to FCC on silica gel EtOAc-Hex (10:90, v/v) to afford ethyl indolyl-3-propanoate (**321,** 545 mg, 2.51 mmol, 96%) as pale yellow solid (Pedras, Minic et al., 2010b). To a solution of ethyl ester **321** (200 mg, 0.926 mmol) in dry toluene (2 ml) under argon atmosphere was added DIBAl-H (1.5 M, 800 µl, 1.20 mmol) at -78 °C and the temperature was gradually raised to -60 °C while stirring. After 10 min, the reaction mixture was quenched with 1N HCl (10 ml) and extracted in EtOAc (3 x 25 ml).The combined organic layers were dried over Na_2SO_4 and concentrated to afford a residue that was subjected to FCC (SiO_2, EtOAc-Hex, 30:70, v/v) to afford indole-3-propanaldehyde (**322,** 160 mg, 90%). Conversion of the aldehyde **322** (150 mg, 0.78 mmol) to dithiocarbamate in three consecutive steps as in the case of brassinin (**17**) yielded methyl 3-(3-indolyl)propyldithiocarbamate (**325**, 88 mg, 0.33 mmol) as an off-white solid in 42% yield over 3 steps. The analytical data matched with published values (Gaspari, Banerjee et al., 2006).

HPLC t_R = 14.7 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.00 (br s, NH, 1H), 7.60 (m, 1H), 7.37 (d, *J* = 8 Hz, 1H), 7.23 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.15 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.00 (s, 1H), 6.91 (br s, NH), 3.80 (q, *J* = 7 Hz, 2H), 2.86 (t, *J* = 7 Hz, 2H), 2.54 (s, 3H), 2.1 (m, 2H) and signals due to a minor rotamer (ca. 30%) 3.50 (q, *J* = 7 Hz), 2.68 (s) (Gaspari et al., 2006).

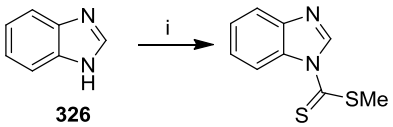
^{13}C NMR (125 MHz, $CDCl_3$): δ 199.7, 136.6, 127.3, 122.3, 121.8, 119.6, 118.9, 115.3, 111.5, 47.2, 28.3, 22.7, 18.0 and signals due to a minor rotamer at 46.0, 28.9, 18.7.

FTIR (KBr) ν_{max} cm^{-1}: 3408, 3323, 2918, 1504, 1456, 1336, 1094, 940, 744.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 200, 220, 272.

HRMS-EI *m/z*: measured 264.0752 ($[M]^+$, calcd. 264.0755 for $C_{13}H_{16}N_2S_2$). MS (EI) *m/z* (% relative int.): 264 [M^+] (11), 231 (26), 216 (36), 184 (12), 156 (15), 130 (100).

4.3.2.6 Methyl benzimidazolyldithiocarbamate (327)



326 **327**

Reagents and conditions: (i) NaH, THF, 0 °C; (ii) CS_2, CH_3I, 72%.

A solution of benzimidazole (**324**, 100 mg, 0.85 mmol) in THF (1 ml) was added to a stirred suspension of sodium hydride (26 mg, 1.1 mmol)) in THF (1 ml) and stirred at r.t for 5 min. Next, CS_2 (61 µl, 1.02 mmol) followed by MeI (80 µl, 1.27 mmol) were added and the reaction mixture was stirred at r.t. After 20 min, the reaction mixture was diluted with ice cold water and extracted with EtOAc (2 × 30 ml). The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure to yield a residue which was purified by FCC (SiO_2; EtOAc-Hex, 20:80; v/v) to afford the product methyl benzimidazolyldithiocarbamate (**327,** 126 mg, 0.61 mmol) as a yellow solid in 72% yield.

HPLC t_R = 11.2 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.88 (s, 1H), 8.54 (d, *J* = 7.5 Hz, 1H), 7.81 (d, *J* = 8.5 Hz, 1H), 7.44-7.38 (m, 2H), 2.85 (s, 3H).

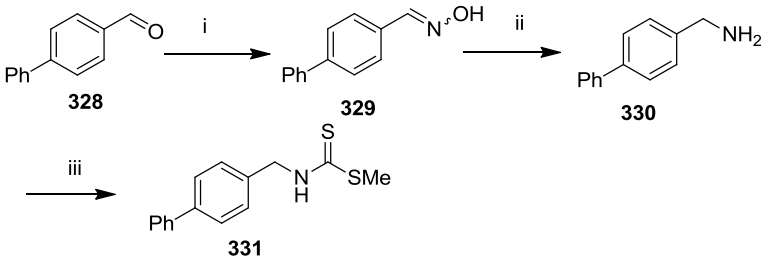
^{13}C NMR (125 MHz, $CDCl_3$): δ 199.1, 145.5, 141.8, 133.0, 125.9, 125.3, 121.2, 115.8, 20.1.

FTIR (KBr) ν_{max} cm^{-1}: 3072, 1511, 1446, 1350, 1277, 1221, 1182, 1048, 1018, 825, 742.

UV (HPLC, $CH_3OH–H_2O$) λ_{max} (nm): 210, 262, 315.

HRMS-EI *m/z*: measured 208.0127 ($[M]^+$, calcd. 208.0129 for $C_9H_8N_2S_2$). MS (EI) *m/z* (% relative int.): 208 [M^+] (100), 161 (64), 134 (27), 91 (100), 63 (8).

4.3.2.7 Methyl 4-biphenyldithiocarbamate (331)



Reagents and conditions: (i) $NH_2OH.HCl$, Na_2CO_3, $EtOH/H_2O$, 97%; (ii) $NaBH_4$, $NiCl_2.6H_2O$, MeOH, 91%; (iii) Pyr, Et_3N, CS_2, CH_3I, 72%.

A solution of $NH_2OH.HCl$ (757 mg, 10.97 mmol) and Na_2CO_3 (873 mg, 8.23 mmol) in water (4 ml) was added to a solution of 4-biphenylcaboxaldehyde (**328**, 1000 mg, 5.48 mmol) in ethanol (10 ml) and refluxed for 30 min. The reaction mixture was concentrated to 1/3 volume, diluted with water and extracted in EtOAc (3 × 40 ml). The combined organic extract was dried over Na_2SO_4 and concentrated to afford 4-biphenylcarboxaldoximes (**329,** 1049 mg) in 97% yield. $NaBH_4$ (234 mg, 6.18 mmol) and $NiCl_2.6H_2O$ (245 mg, 1.03 mmol) were added to a cooled solution of the oximes **329** (202 mg, 1.03 mmol) in methanol (8 ml) and the reaction mixture was stirred at r.t. After 15 min, the reaction mixture was diluted with 1% NH_4OH (30 ml) and filtered. The filtrate was extracted with EtOAc (3 × 40 ml) and the organic extract was combined, dried over Na_2SO_4 and concentrated under reduced pressure. The residue

was further separated by FCC (SiO_2; MeOH/DCM/NH_4OH) to afford 4-biphenylmethanamime (**330,** 170 mg, 0.93 mmol) in 91% yield. CS_2 (68 µl, 1.13 mmol) followed by MeI (88 µl, 1.40 mmol) were added to a solution of amine **330** (0 mg, 0.94 mmol) and triethylamine (256 µl, 1.88 mmol) in pyridine (2 ml) and stirred at r.t. After 30 min, the reaction mixture was diluted with toluene (2 × 10 ml) and concentrated under reduced pressure. The residue was loaded on the column of silica gel and eluted with EtOAc-Hex (20:80, v/v) to afford the product **331** (185 mg, 72%, 0.68 mmol).

HPLC t_R = 23.9 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.60 (d, *J* = 8 Hz, 4H), 7.48 (dd, *J* = 8, 8 Hz, 2H), 7.42-7.38 (m, 3H), 7.27 (br s, NH), 4.98 (d, *J* = 5 Hz, 2H), 2.69 (s, 3H). Minor rotamers were found at δ 4.66 and 2.72.

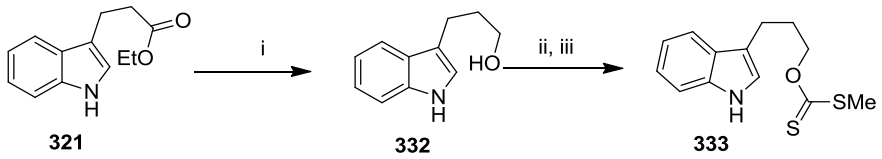
^{13}C NMR (125 MHz, $CDCl_3$): δ 199.5, 141.3, 140.7, 135.4, 129.1, 128.9, 127.8, 127.7, 127.3, 51.1, 18.5.

FTIR (KBr) ν_{max} cm^{-1}: 3339, 3224, 2919, 1486, 1328, 1088, 926, 761, 697.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 204, 260.

HRMS-EI *m/z*: measured 273.0646 ([M] $^+$, calcd. 273.0646 for $C_{15}H_{15}NS_2$). MS (EI) *m/z* (% relative int.): 273 [M^+] (16), 225 (20), 167 (100), 152 (9).

4.3.2.8 Methyl indolyl-3-propyldithiocarbonate (333)



321 332 333

Reagents and conditions: (i) $LiAlH_4$, THF, 0 °C, 95%; (ii) NaH, THF, 0 °C; (iii) CS_2, CH_3I, 73%.

LAH (116 mg, 3.056 mmol) was added in portions to a cooled solution of ester **321** (550mg, 2.546 mmol) in dry THF (5 ml) and the reaction mixture was stirred at room temperature for 40 min. The reaction mixture was quenched with 1M NaOH solution (5 ml) and the precipitate was vacuum filtered. The filtrate was extracted with EtOAc (2 x

50 ml) and the combined organic extract was dried over Na_2SO_4, and concentrated using a rotary evaporator under reduced pressure. The residue was separated by FCC (SiO_2; 30% EtOAc/Hex) to afford 3-indolylpropanol (**332,** 420 mg, 2.40 mmol**)** as pale yellow oil in 95% yield. A solution of 3-(3-indolyl)propanol (**332,** 30 mg, 0.17 mmol) in THF (0.5 ml) was added to a suspension of sodium hydride (6 mg, 0.21 mmol) in THF (2 ml) and stirred at r.t for 5 min. Next, CS_2 (19 µl, 0.31 mmol) followed by MeI (20 µl, 0.31 mmol) were added and the reaction mixture was stirred at r.t. After 20 min, the reaction mixture was diluted with ice-cold water and extracted with EtOAc (2 × 40 ml). The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure to yield a residue that was purified by FCC (SiO_2; EtOAc-Hex, 20:80, v/v) to afford methyl 3-(3-indolyl)propyldithiocarbonate (**333**, 33 mg, 0.12 mmol) as a yellow solid in 72% yield.

HPLC t_R = 21.4 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.94 (br s, NH), 7.64 (d, *J* = 8 Hz, 1H), 7.38 (d, *J* = 8 Hz, 1H), 7.25 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.17 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.01 (s, 1H), 4.69 (t, *J* = 6.5 Hz, 2H), 2.94 (t, *J* = 7.5 Hz, 2H), 2.60 (s, 3H), 2.25 (m, 2H).

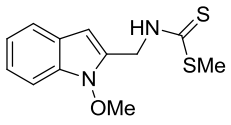
^{13}C NMR (125 MHz, $CDCl_3$): δ 216.1, 136.5, 127.4, 122.2, 121.7, 119.4, 118.9, 115.1, 111.3, 73.8, 28.7, 21.6, 19.1.

FTIR (KBr) ν_{max} cm^{-1}: 3414, 2921, 1456, 1223, 1056, 742.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 220, 278.

HRMS-EI *m/z*: measured 265.0587 ([M] $^+$, calcd. 265.0595 for $C_{13}H_{15}NOS_2$). MS (EI) *m*/*z* (% relative int.): 265 [M^+] (33), 232 (52), 218 (32), 157 (52), 130 (100).

4.3.2.9 1-Methoxyisobrassinin (342)



342

Compound **342** was synthesized as previously reported (Pedras, Suchy et al., 2006) and as described in section **4.3.1.3**.

HPLC t_R = 24.6 min (method A).

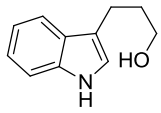
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.57 (d, *J* = 8 Hz, 1H), 7.43 (d, *J* = 8 Hz, 1H), 7.28 (dd, *J* = 8, 8 Hz, 1H), 7.20 (br s, NH), 7.14 (dd, *J* = 8, 8 Hz, 1H), 6.38 (s, 1 H), 5.14 (d, *J* = 5 Hz, 2H), 4.10 (s, 3H), 2.67 (s, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 200.0, 133.0, 131.0, 123.7, 123.1, 121.4, 120.8, 108.5, 98.9, 65.9, 42.3, 18.5.

HRMS-EI *m/z*: measured 266.0543 ($[M]^+$, calcd. 266.0548 for $C_{12}H_{14}N_2OS_2$). MS (EI) *m/z* (% relative int.): 266 [M^+] (9), 235 (100), 218 (23), 160 (54), 129 (82), 91 (43).

4.3.3 Synthesis of metabolites

4.3.3.1 3-(3-Indolyl)propanol (332)



332

Synthesized as described earlier (**4.3.2.9**) in 95% yield.

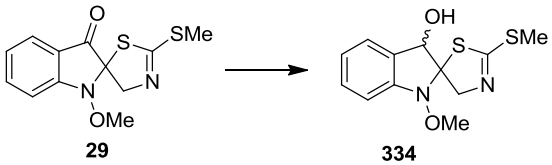
HPLC t_R = 7.2 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.10 (br s, NH), 7.65 (d, *J* = 8 Hz, 1H), 7.36 (d, *J* = 8 Hz, 1H), 7.25 (dd, *J* = 7, 8 Hz, 1H), 7.16 (dd, *J* = 7, 8 Hz, 1H), 6.96 (s, 1H), 3.74 (t, *J* = 6.5 Hz, 2H), 2.88 (t, *J* = 7.5 Hz, 2H), 2.01 (quint, 2H), 1.81 (br s, OH).

^{13}C NMR (125 MHz, $CDCl_3$): δ 136.6, 127.6, 122.1, 121.5, 119.3, 119.0, 116.0, 111.3, 62.7, 33.1, 21.5.

HRMS-EI *m/z*: measured 175.0988 ($[M]^+$, calcd. 175.0991 for $C_{11}H_{13}NO$). MS (EI) *m/z* (% relative int.): 175 [M^+] (33), 144 (5), 130 (100), 78 (1).

4.3.3.2 Dihydroerucalexin (334)



Reagents and conditions: (i) $NaBH_4$, MeOH, 0 °C, 60%.

$NaBH_4$ (1 mg, 0.032 mmol) was added to a solution of erucalexin (**29**, 15 mg, 0.053 mmol) in methanol (1 ml) at 0 °C and stirred at the same temperature. After 5 min, the reaction mixture was diluted with water (1 ml) and directly loaded onto a reversed phase column (C18 silica gel) and eluted with H_2O-CH_3CN (60:40, v:v) to afford the mixture of diastereomeric products **334** (9 mg, 0.03 mmol, 60% yield) as a white solid in 3:1 ratio.

HPLC t_R = 9.2 min (major) and 11.9 min (minor) (method A).

Major isomer: ^{1}H NMR (500 MHz, CD_3CN): δ 7.31-7.23 (m, 2H), 7.00 (ddd, *J* = 8, 8, 1 Hz, 1H), 6.93 (d, *J* = 8 Hz, 1H), 4.94 (s, 1H), 4.65 (d, *J* = 16 Hz, 1H), 4.26 (d, *J* = 16 Hz, 1H), 4.09 (br s, OH), 3.86 (s, 3H), 2.51 (s, SMe, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 164.9, 150.7, 130.4, 124.5, 123.9, 112.5, 74.3, 70.2, 66.8, 65.3, 15.5.

Minor isomer: ^{1}H NMR (500 MHz, CD_3CN): δ 7.31-7.23 (m, 2H), 7.00 (ddd, *J* = 8, 8, 1 Hz, 1H), 6.93 (d, *J* = 8 Hz, 1H), 5.24 (s, 1H), 4.49 (d, *J* = 16.5 Hz, 1H), 4.45 (d, *J* = 16.5 Hz, 1H), 4.09 (br s, OH), 3.88 (s, OMe, 3H), 2.51 (s, SMe, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 163.8, 152.0, 131.1, 130.5, 126.3, 124.1, 113.0, 103.9, 76.3, 66.7, 15.5.

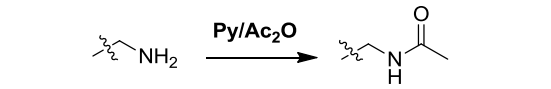
FTIR (KBr) ν_{max} cm^{-1}: 3317, 2929, 1614, 1570, 1463, 1193, 994, 954, 758.

UV (HPLC, CH_3CN–H_2O) λ_{max} (nm): 208, 240, 285.

HRMS-EI *m/z*: measured 282.0502 ([M] $^+$, calcd. 282.0497 for $C_{12}H_{14}N_2O_2S_2$). MS (EI) *m*/*z* (% relative int.): 282 [M^+] (12), 250 (51), 191 (100), 177 (68), 149 (22), 132 (57), 117 (34), 87 (41), 72 (48).

4.3.3.3 Synthesis of compounds 340, 344, 346, 348 and 349

General procedure:

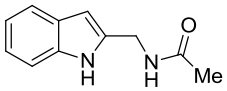


155, 324, 330, 339, 343 **340, 344, 346, 348, 349**

Reagents and conditions: (i) Pyr, Ac_2O, DCM, 0 °C, 74-90%.

Acetic anhydride (1.1 eq) was added to a solution of amines **155**, **324**, **330**, **339** or **343** (1 eq) and pyridine (1.5 eq) in DCM (2 ml) at 0 °C and stirred at r.t. for 10-30 min. After complete conversion of the starting material, the reaction mixture was diluted with toluene (5 ml) and concentrated using a rotary evaporator to afford crude substituted acetamides which were separated by FCC (SiO_2; EtOAc) to afford compounds **340**, **344**, **346**, **348** and **349**.

4.3.3.4 N_b-Acetyl-2-indolylmethanamine (340)



340

Acetylation of indolyl-2-methanamine (**339**, 30 mg, 0.20 mmol) using the above procedure afforded compound **340** (29 mg, 0.15 mmol) in 75% yield.

HPLC t_R = 6.2 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.05 (br s, NH), 7.57 (d, *J* = 8 Hz, 1H), 7.33 (d, *J* = 8 Hz, 1H), 7.17 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.09 (dd, *J* = 7.5, 7.5 Hz, 1H), 6.32 (s, 1H), 6.18 (br s, NHAc), 4.45 (d, *J* = 6 Hz, 2H), 2.00 (s, 3H).

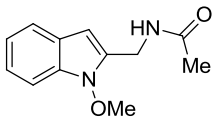
^{13}C NMR (125 MHz, $CDCl_3$): δ 171.9, 136.6, 127.8, 122.2, 120.5, 119.9, 111.3, 100.7, 37.6, 23.2.

FTIR (KBr) ν_{max} cm^{-1}: 3392, 3269, 3060, 1651, 1531, 1456, 1423, 1288, 749.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 219, 272.

HRMS-EI *m/z*: measured 188.0947 ([M] $^{+}$, calcd. 188.0950 for $C_{11}H_{12}N_2O$). MS (EI) *m/z* (% relative int.): 188 [M^{+}] (100), 145 (46), 130 (40), 118 (51).

4.3.3.5 N_b-Acetyl-1-methoxy-2- indolylmethanamine (344)



344

Acetylation of 1-methoxy-2-indolylmethanamine (**343**, 209 mg, 1.19 mmol) using the general procedure afforded compound **344** (223 mg, 1.02 mmol) in 84% yield.

HPLC t_R = 8.9 min (method C).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.52 (d, *J* = 8 Hz, 1H), 7.39 (d, *J* = 8 Hz, 1H), 7.24 (dd, *J* = 8, 8 Hz, 1H), 7.11 (dd, *J* = 8, 8 Hz, 1H), 6.32 (br s, NHAc), 6.24 (s, 1H), 4.58 (d, *J* = 6 Hz, 2 H), 4.04 (s, 3H), 1.98 (s, 3H).

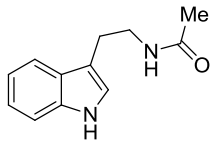
^{13}C NMR (125 MHz, $CDCl_3$): δ 170.3, 133.3, 132.7, 123.7, 122.5, 121.0, 120.5, 108.2, 97.4, 65.5, 34.7, 23.2.

FTIR (KBr) ν_{max} cm^{-1}: 3275, 3060, 2940, 1655, 1541, 1452, 1288, 971, 744.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 220, 270.

HRMS-EI *m/z*: measured 218.1050 ([M] $^{+}$, calcd. 218.1055 for $C_{12}H_{14}N_2O_2$). MS (EI) *m/z* (% relative int.): 218 [M^{+}] (28), 187 (44), 169 (27), 145 (100), 129 (12), 118 (70), 100 (9), 89 (14).

4.3.3.6 N_b-Acetyltryptamine (346)



346

Acetylation of tryptamine (**155**, 100 mg, 0.62 mmol) using above procedure afforded compound **346** (115 mg, 0.57 mmol) in 91% yield (Pedras and Okanga, 2000).

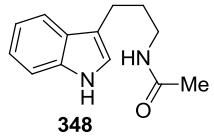
HPLC t_R = 18.3 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.66 (br s, NH), 7.60 (d, *J* = 8 Hz, 1H), 7.37 (d, *J* = 8 Hz, 1H), 7.19 (dd, *J* = 8, 7.5 Hz, 1H), 7.13 (dd, *J* = 8, 7.5 Hz, 1H), 7.00 (s, 1H), 5.80 (br s, NH), 3.58 (q, *J* = 7 Hz, 2H), 2.97 (t, *J* = 7 Hz, 2H), 1.91 (s, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 170.5, 136.6, 127.4, 122.4, 122.2, 119.5, 118.7, 112.8, 111.5, 40.1, 25.4, 23.4.

HRMS-EI *m/z*: measured 202.1109 ([M] $^+$, calcd. 202.1106 for $C_{12}H_{14}N_2O$). MS (EI) *m/z* (% relative int.): 202 [M^+] (20), 144 (12), 143 (100), 130 (98).

4.3.3.7 N_b-Acetyl-3-indolylpropanamine (348)



348

Acetylation of 3-indolylpropanamine (**324,** 150 mg, 0.867mmol) using the above procedure afforded compound **348** (138 mg, 0.64 mmol) in 74% yield.

HPLC t_R = 21.4 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.54 (br s, NH), 7.58 (d, *J* = 8 Hz, 1H), 7.34 (d, *J* = 8 Hz, 1H), 7.20 (dd, *J* = 8, 7 Hz, 1H), 7.12 (dd, *J* = 8, 7 Hz, 1H), 6.90 (s, 1H), 5.86 (br s, NHAc), 3.28 (m, 2H), 2.77 (t, *J* = 7 Hz, 2H), 1.91 (s, 3H), 1.89 (quint, *J* = 7 Hz, 2H).

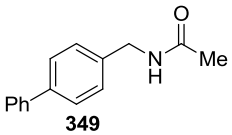
^{13}C NMR (125 MHz, $CDCl_3$): δ 170.6, 136.6, 127.4, 121.9, 121.8, 119.1, 118.8, 115.2, 111.5, 39.7, 29.8, 23.3, 22.7.

FTIR (KBr) ν_{max} cm^{-1}: 3403, 3283, 2930, 1652, 1550, 1456, 743.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 221, 282.

HRMS-EI *m/z*: measured 216.1266 ([M] $^+$, calcd. 216.1263 for $C_{13}H_{16}N_2O$). MS (EI) *m/z* (% relative int.): 216 [M^+] (39), 157 (35), 144 (37), 130 (100).

4.3.3.8 N-Acetyl-4-biphenylmethanamine (349)



349

Acetylation of 4-biphenylmethanamine (**330,** 225 mg, 1.24 mmol) using the above procedure afforded compound **349** (251 mg, 1.11 mmol) in 90% yield.

HPLC t_R = 18.3 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.64 (d, *J* = 8 Hz, 2H), 7.61 (d, *J* = 8 Hz, 2H), 7.46 (dd, *J* = 7, 8 Hz, 2H), 7.36 (m, 3H), 6.85 (br s, NH), 4.36 (d, *J* = 6 Hz, 2H), 1.92 (s, 3H).

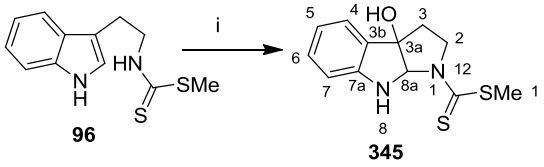
^{13}C NMR (125 MHz, $CDCl_3$): δ 171.0, 142.0, 140.9, 140.3, 130.3, 129.3, 128.7, 128.3, 128.2, 43.6, 23.4.

FTIR (KBr) ν_{max} cm^{-1}: 3292, 3091, 1638, 1554, 1443, 1373, 1294, 1094, 756.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 206, 252.

HRMS-EI *m/z*: measured 225.1148 ([M] $^+$, calcd. 225.1154 for $C_{15}H_{15}NO$). MS (EI) *m/z* (% relative int.): 225 [M^+] (100), 182 (66), 167 (34), 106 (20).

4.3.3.9 Methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (345)



Reagents and conditions: (i) Rose Bengal, 5% Pyr/MeOH, hν, 53% (Pedras and Okanga, 2000).

Compound **345** was prepared according to a published procedure (Pedras and Okanga, 2000). Rose bengal (16 mg, 0.016 mmol) was added to a solution of **96** (40 mg, 0.16 mmol) in 5% Pyr/MeOH (6 ml) in a pyrex tube and the reaction mixture was cooled in an icebath. Next, the reaction medium was irradiated with 100 W halogen bulb under continuous bubbling of air. After 2 hrs, the reaction mixture was transferred to round bottom flask, dimethylsulfide (300 µl) was added and the reaction mixture was stirred at r.t. After 1 h, the reaction mixture was diluted with toluene (10 ml) and concentrated under reduced pressure. The residue was separated by FCC (SiO_2; DCM-Et_2O, 1:1, v/v) to afford **345** (16 mg, 0.06 mmol) in 53% yield based on recovered starting material.

HPLC t_R = 11.5 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.33 (d, *J* = 7.5 Hz, 1H), 7.20 (dd, *J* = 8, 7.5 Hz, 1H), 6.83 (dd, *J* = 7.5, 7.5 Hz, 1H), 6.65 (d, *J* = 8, 7.5 Hz, 1H), 5.76 (s, 1H), 4.14 (m, 1H), 3.36 (m, 1H), 2.65 (s, 3H), 2.65-2.55 (m, 2H).

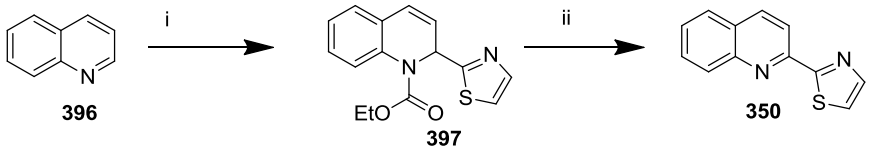
^{13}C NMR (125 MHz, $CDCl_3$): δ 197.6, 149.2, 131.1, 128.8, 123.7, 119.7, 110.4, 87.3, 86.4, 50.3, 36.1, 19.5.

HRMS-EI *m/z*: measured 266.0544 ($[M]^+$, calcd. 266.0548 for $C_{12}H_{14}N_2OS_2$). MS (EI) *m/z* (% relative int.): 266 [M^+] (100), 251 (8), 219 (19), 186 (42), 160 (41), 133 (56), 91 (14), 69 (40).

4.4 Brassinin detoxification inhibitors

4.4.1 Synthesis of quinoline derivatives

4.4.1.1 2-(2-Thiazolyl)quinoline (350)



Reagents and conditions: (i) $ClCO_2Et$, DCM, 2-(trimethylsilyl)thiazole, r.t., 25%; (ii) *o*-chloranil, C_6H_6, reflux, 52% (Dondoni, Dall' Occo et al., 1984).

Ethylchloroformate (73 mg, 0.77 mmol) was added to a solution of quinoline (**396**, 100 mg, 0.77 mmol) in DCM at 0 ºC and the reaction mixture was stirred at r.t. After 30 min, 2-(trimethylsilyl)thiazole (122 mg, 0.77 mmol) was added and the reaction mixture was stirred at r.t. for 3 h. The reaction mixture was concentrated under reduced pressure and the residue was separated by FCC (SiO_2, DCM/Hex) to afford 2-(2-thiazolyl)-1(2H)-quinolinecarboxylic acid ethyl ester (**397**, 55 mg) as a pale yellow solid in 25% yield. *o*-Chloranil (54 mg, 0.22 mmol) was added to a solution of compound **397** (55 mg, 0.202 mmol) in benzene (3 ml) and the reaction mixture was refluxed at 80 ºC. After 5 h, the reaction mixture was diluted with 5% NaOH (10 ml) and extracted with DCM (2 × 30 ml). The combined organic extract was dried over

Na_2SO_4 and concentrated to yield the crude which was separated by FCC (SiO_2, DCM/Hex) to afford the product **350** (Dondoni, Dall' Occo et al., 1984) as a white solid (23 mg, 0.11 mmol) in 52% yield.

HPLC t_R = 15.2 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.35 (d, *J* = 8.5 Hz, 1H), 8.27 (d, *J* = 8.5 Hz, 1H), 8.15 (d, *J* = 8.5 Hz, 1H), 7.99 (d, *J* = 3 Hz, 1H), 7.85 (d, *J* = 8 Hz, 1H), 7.75 (ddd, *J* = 8, 8, 1 Hz, 1H), 7.57 (ddd, *J* = 8, 8, 1 Hz, 1H), 7.52 (d, *J* = 3 Hz, 1H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 144.3, 137.3, 131.9, 129.7, 128.8, 127.9, 127.3, 125.9, 122.2, 118.0, 99.4, 99.3.

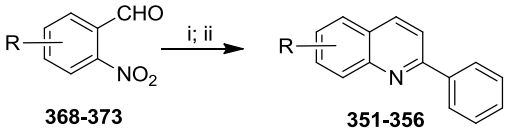
FTIR (KBr) ν_{max} cm^{-1}: 3072, 1593, 1504, 1427, 1311, 1116, 1002, 938, 834, 755.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 220, 240, 275, 330.

HRMS-EI *m/z*: measured 212.0412 ($[M]^+$, calcd. 212.0408 for $C_{12}H_7N_2S$). MS (EI) *m/z* (% relative int.): 212 [M^+] (100), 186 (14), 162 (34), 151 (20), 128 (11), 100 (35).

4.4.1.2 Synthesis of compounds 351-356

General procedure



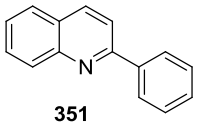
368-373 **351-356**

Reagents and conditions: (i) Fe/HCl, EtOH; (ii) KOH, $C_6H_5C(O)Me$, 42- 83%.

To a solution of o-nitrobenzaldehyde (1 mmol) in EtOH (3 ml) was added iron Fe powder (4 mmol) followed by 0.1 N HCl (0.05 mmol) and the reaction mixture was refluxed at 95 ºC for 40 min. After complete consumption of the starting material, acetophenone (1 mmol) followed by KOH (1.2 mmol) were added and the mixture was heated at 95 ºC for 3 h (Li, Ahmed et al., 2007). The reaction mixture was filtered, neutralized with 10% HCl (3 ml) and extracted in EtOAc (2 × 30 ml). The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure to

afford the crude mixture which was separated by FCC (SiO_2; DCM/Hex) to afford **351-356** in 42-83% yields.

2-Phenylquinoline (351)



351

Compound **351** was prepared starting from compound **369** (151 mg, 1.00 mmol) according to the above procedure in 83% yield (171 mg, 0.83 mmol) (Li, Ahmed et al., 2007). Analytical data matched with the published values (Movassaghi and Hill, 2006).

HPLC t_R = 18.8 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.24 (d, *J* = 8.5 Hz, 1H), 8.18 (m, 3H), 7.89 (d, *J* = 8.5 Hz, 1H), 7.84 (d, *J* = 8 Hz, 1H), 7.74 (dd, *J* = 7.5, 8 Hz, 1H), 7.54 (dd, *J* = 7.5, 8 Hz, 3H), 7.48 (m, 1H).

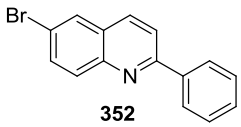
^{13}C NMR (125 MHz, $CDCl_3$): δ 157.6, 148.5, 139.9, 136.9, 129.9, 129.8, 129.5, 129.0, 127.8, 127.7, 127.4, 126.5, 119.2.

FTIR (KBr) ν_{max} cm^{-1}: 3055, 1617, 1597, 1554, 1490, 1446, 1320, 1284, 829, 765, 691.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 210, 259, 322.

HRMS-EI *m/z*: measured 205.0890 ([M] $^+$, calcd. 205.0891 for $C_{15}H_{11}N$). MS (EI) *m/z* (% relative int.): 205 [M^+] (100), 176 (5), 149 (5), 102 (14), 76 (6).

6-Bromo-2-phenylquinoline (352)



352

3-Bromobenzaldehyde (**367**, 558 mg, 3.01 mmol) was added drpwise to a cooled mixture of HNO_3/H_2SO_4 (0.3ml/3 ml) at 0 ºC and the reaction mixture was stirred at 5 ºC. After 1 h, the reaction mixture was poured into ice-cold water (20 ml) and the

precipitate was filtered. The crude product was purified by FCC (SiO_2; 20% EtOAc/Hex) to afford compound **368** (470 mg, 2.04 mmol, 68%) as a brown solid (Alford and Schofield, 1952). Next, compound **352** was prepared starting from compound **368 (**59 mg, 0.25 mmol) according to the general procedure described above in 64% yield **(**47 mg, 0.16 mmol). Analytical data matched published values (Huo, Gridnev et al., 2010).

HPLC t_R = 20.5 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.20-8.12 (m, 3H), 8.05 (d, *J* = 9 Hz, 1H), 8.00 (d, *J* = 2 Hz, 1H), 7.92 (d, *J* = 9 Hz, 1H), 7.80 (dd, *J* = 2, 9 Hz, 1H), 7.55 (dd, *J* = 7, 7 Hz, 2H), 7.50 (m, 1H).

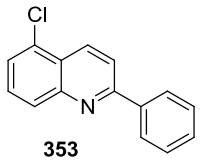
^{13}C NMR (125 MHz, $CDCl_3$): δ 157.9, 147.1, 139.5, 135.9, 133.3, 131.7, 129.86, 129.7, 129.1, 128.5, 127.7, 120.3, 120.0.

FTIR (KBr) ν_{max} cm^{-1}: 1595, 1542, 1483, 1278, 887, 875, 830, 786, 758, 695.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 220, 257, 325.

HRMS-EI *m/z*: measured 284.9987 ([M^+], calcd. 284.9976 [100%] for $C_{15}H_{10}{}^{81}BrN$), 283.0001 ([M^+], calcd. 282.9997 for $C_{15}H_{10}{}^{79}BrN$). MS (EI) *m/z* (% relative int.): 285 (100), 283 [M^+] (99), 281 (29), 204 (41), 203 (33), 102 (26).

5-Chloro-2-phenylquinoline (353)



353

Compound **353** was prepared starting from compound **370** (200 mg, 1.07 mmol) according to the above procedure in 84% yield (215 mg, 0.90 mmol).

HPLC t_R = 24.4 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.63 (d, *J* = 9 Hz, 1H), 8.19 (m, 2H), 8.11 (d, *J* = 8 Hz, 1H), 7.99 (d, *J* = 9 Hz, 1H), 7.68-7.59 (m, 2H), 7.56 (m, 2H), 7.50 (dd, *J* = 7, 7 Hz, 1H).

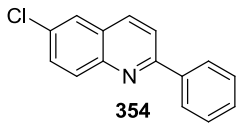
^{13}C NMR (125 MHz, $CDCl_3$): δ 158.1, 149.2, 139.2, 133.8, 131.4, 129.9, 129.5, 129.2, 129.1, 127.8, 126.5, 125.5, 119.9.

FTIR (KBr) ν_{max} cm^{-1}: 3058, 1611, 1593, 1580, 1547, 1486, 1461, 1396, 1316, 1278, 1201, 1025, 960, 814, 775, 692, 671.

UV (HPLC, $CH_3OH–H_2O$) λ_{max} (nm): 208, 262.

HRMS-EI *m/z*: measured 241.0481 ([M] $^+$, calcd. 241.0472 [32%] for $C_{15}H_{10}N^{37}Cl$), 239.0507 ([M] $^+$, calcd. 239.0502 for $C_{15}H_{10}NCl$). MS (EI) *m/z* (% relative int.): 241 (32), 239 [M^+] (100), 204 (33), 102 (11).

6-Chloro-2-phenylquinoline (354)



354

Compound **354** was prepared starting from compound **371** (200 mg, 1.07 mmol) according to the above procedure in 63% yield (161 mg, 0.67 mmol). Analytical data matched with the published values (Shi, Rong et al., 2005).

HPLC t_R = 22.2 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.20-8.09 (m, 4H), 7.91 (d, *J* = 9 Hz, 1H), 7.82 (d, *J* = 2 Hz, 1H), 7.67 (dd, *J* = 2, 9 Hz, 1H), 7.55 (t, *J* = 7.5 Hz, 2H), 7.49 (dd, *J* = 7, 7.5 Hz, 1H).

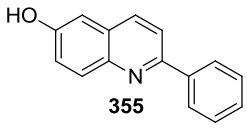
^{13}C NMR (125 MHz, $CDCl_3$): δ 157.8, 146.9, 139.4, 136.0, 132.1, 131.6, 130.8, 129.8, 129.1, 128.0, 127.7, 126.3, 120.0.

FTIR (KBr) ν_{max} cm^{-1}: 3060, 1596, 1548, 1484, 1445, 1320, 1280, 1192, 1130, 1075, 945, 886, 876, 832, 782, 755, 695.

UV (HPLC, $CH_3OH–H_2O$) λ_{max} (nm): 215, 255, 325.

HRMS-EI *m/z*: measured 241.0469 ([M] $^+$, calcd. 241.0472 [33%] for $C_{15}H_{10}N^{37}Cl$), 239.0496 ([M] $^+$, calcd. 239.0502 for $C_{15}H_{10}NCl$). MS (EI) *m/z* (% relative int.): 241 (33), 239 [M^+] (100), 204 (33), 102 (9).

6-Hydroxy-2-phenylquinoline (355)



355

Compound **355** was prepared starting from compound **372** (200 mg, 1.19 mmol) according to the above procedure in 42% yield (112 mg, 0.51 mmol). Analytical data matched with the published values (Melendez Gomez, Kouznetsov et al., 2008).

HPLC t_R = 14.2 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.48-7.45 (m, 4H), 7.40 (d, *J* = 2 Hz , 1H), 7.33 (dd, *J* = 2, 2.5 Hz, 1H), 7.31 (dd, *J* = 2, 2.5 Hz, 1H), 7.28 (dd, *J* = 2, 2.5 Hz, 1H), 7.24 (d, *J* = 2 Hz, 1H), 6.84 (br s, OH).

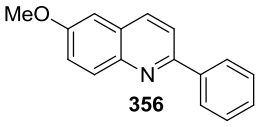
^{13}C NMR (125 MHz, $CDCl_3$): δ 155.5, 153.9, 140.0, 135.5, 131.7, 129.2, 129.0, 128.4, 127.6, 121.7, 119.7, 109.1.

FTIR (KBr) ν_{max} cm^{-1}: 3500-2500 (br), 1619, 1561, 1452, 1393, 1349, 1228, 1131, 928, 859, 826, 756.

UV (HPLC, $CH_3OH–H_2O$) λ_{max} (nm): 215, 260, 342.

HRMS-EI *m/z*: measured 221.0841 ([M] $^+$, calcd. 221.0841 for $C_{15}H_{11}NO$). MS (EI) *m/z* (% relative int.): 221 [M^+] (100), 204 (6), 191 (10), 111 (6).

6-Methoxy-2-phenylquinoline (356)



356

Compound **356** was prepared starting from compound **373** (150 mg, 0.82 mmol) according to the above procedure in 60% yield (115 mg, 0.49 mmol). Analytical data matched published values (Movassaghi and Hill, 2006).

HPLC t_R = 19.3 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.19-8.05 (m, 4H), 7.85 (d, *J* = 9 Hz , 1H), 7.53 (dd, *J* = 7.5, 7 Hz, 1H), 7.45 (dd, *J* = 7, 7.5 Hz, 1H), 7.40 (dd, *J* = 9, 3 Hz, 1H), 7.10 (d, *J* = 3 Hz, 1H), 3.96 (s, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 157.9, 155.3, 144.7, 140.1, 135.7, 131.5, 129.2, 129.0, 128.4, 127.5, 122.5, 119.4, 105.3, 55.7.

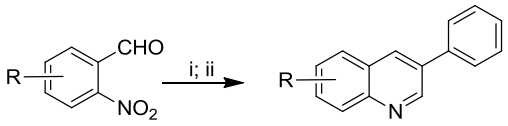
FTIR (KBr) ν_{max} cm^{-1}: 3006, 1620, 1597, 1557, 1492, 1340, 1251, 1228, 1163, 1021, 860, 832, 762.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 216, 256, 336.

HRMS-EI *m/z*: measured 235.0994 ($[M]^+$, calcd. 235.0997 for $C_{16}H_{13}NO$). MS (EI) *m*/*z* (% relative int.): 235 [M^+] (100), 220 (21), 192 (34).

4.4.1.3 Synthesis of compounds 357-361

General procedure

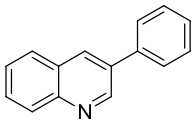


368-373 **357-361**

Reagents and conditions: (i) Fe/HCl, EtOH; (ii) KOH, $PhCH_2CHO$, 39- 80%.

To a solution of o-nitrobenzaldehyde (1 mmol) in EtOH (3 ml) was added iron powder (4 mmol) followed by 0.1 N HCl (0.05 mmol) and the reaction mixture was refluxed at 95 ºC for 40 min. After consumption of the starting material, phenylacetaldehyde (1 mmol) followed by KOH (1.2 mmol) were added and the the mixture was heated at 95 ºC for 3 h (Li et al., 2007). The reaction mixture was filtered, neutralized with 10% HCl (3 ml) and extracted with EtOAc (2 × 30 ml). The combined organic extract was dried over Na_2SO_4 and concentrated to yield the crude which was purified by FCC (SiO_2, DCM/Hex) to afford the products **358**-**362** in 39-80% yields.

3-Phenylquinoline (357)



357

Compound **357** was prepared starting from compound **369** (200 mg, 1.32 mmol) according to the above procedure in 63% yield (172 mg, 0.84 mmol) (Li, Ahmed et al., 2007). Analytical data matched with the published values.

HPLC t_R = 18.1 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.20 (s, 1H), 8.29 (s, 1H), 8.16 (d, *J* = 8.5 Hz , 1H), 7.87 (d, *J* = 8 Hz, 1H), 7.80-7.66 (m, 3H), 7.63-7.50 (m, 3H), 7.43 (dd, *J* = 7, 7.5 Hz, 1H).

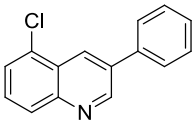
^{13}C NMR (125 MHz, $CDCl_3$): 150.1, 147.5, 138.0, 133.9, 133.3, 129.5, 129.4, 129.2, 128.2, 128.1, 127.6, 127.1, 127.0.

FTIR (KBr) ν_{max} cm^{-1}: 3057, 1492, 1362, 1340, 1125, 1025, 953, 902, 786, 761, 696.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 210, 250, 325.

HRMS-EI *m/z*: measured 205.0884 ($[M]^+$, calcd. 205.0891 for $C_{15}H_{11}N$). MS (EI) *m/z* (% relative int.): 205 [M^+] (100), 176 (8), 149 (8), 76 (7).

5-Chloro-3-phenylquinoline (358)



358

Compound **358** was prepared starting from compound **370** (200 mg, 1.07 mmol) according to the general procedure in 80% yield (207 mg, 0.87 mmol).

HPLC t_R = 22.9 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.18 (d, *J* = 2 Hz , 1H), 8.22 (d, *J* = 2 Hz , 1H), 8.08 (d, *J* = 9 Hz , 1H), 7.88 (d, *J* = 2 Hz, 1H), 7.71 (m, 2H), 7.66 (dd, *J* = 9, 2 Hz, 1H), 7.55 (m, 2H), 7.47 (dd, *J* = 7.5, 7.5 Hz, 1H).

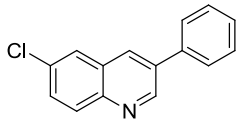
^{13}C NMR (125 MHz, $CDCl_3$): δ 150.8, 148.1, 137.7, 135.0, 131.7, 130.2, 129.5, 129.2, 128.7, 128.6, 127.8, 127.3, 126.4.

FTIR (KBr) ν_{max} cm^{-1}: 3053, 1487, 1456, 1182, 977, 900, 810, 758, 742, 694.

UV (HPLC, $CH_3OH–H_2O$) λ_{max} (nm): 208, 238, 257.

HRMS-EI *m/z*: measured 241.0480 ([M] $^{+}$, calcd. 241.0472 [34%] for $C_{15}H_{10}N^{37}Cl$), 239.0507 ([M] $^{+}$, calcd. 239.0502 for $C_{15}H_{10}NCl$). MS (EI) *m/z* (% relative int.): 241 (34), 239 [M^{+}] (100), 204 (15), 6 (12).

6-Chloro-3-phenylquinoline (359)



359

Compound **359** was prepared starting from compound **371** (200 mg, 1.07 mmol) according to the above procedure in 64% yield (163 mg, 0.68 mmol). Analytical data matched with the published values (Wang, Xin et al., 2009).

HPLC t_R = 21.1 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.23 (d, *J* = 2 Hz, 1H), 8.71 (s, 1H), 8.08 (d, *J* = 7 Hz, 1H), 7.76 (d, *J* = 8 Hz, 2H), 7.69-7.61 (m, 2H), 7.56 (dd, *J* = 7, 8 Hz, 2H), 7.48 (dd, *J* = 7, 8 Hz, 1H).

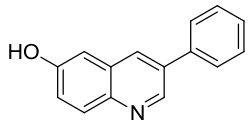
^{13}C NMR (125 MHz, $CDCl_3$): δ 150.8, 148.2, 137.7, 135.0, 131.7, 130.2, 129.5, 129.2, 128.7, 128.6, 127.8, 127.3, 126.4.

FTIR (KBr) ν_{max} cm^{-1}: 3036, 1488, 1452, 1332, 1075, 926, 907, 826, 755, 698.

UV (HPLC, $CH_3OH–H_2O$) λ_{max} (nm): 210, 254, 330.

HRMS-EI *m/z*: measured 241.0474 ([M] $^{+}$, calcd. 241.0472 [34%] for $C_{15}H_{10}N^{37}Cl$), 239.0501 ([M] $^{+}$, calcd. 239.0502 for $C_{15}H_{10}NCl$). MS (EI) *m/z* (% relative int.): 241 (34), 239 [M^{+}] (100), 204 (14), 176 (11), 88 (6).

6-Hydroxy-3-phenylquinoline (360)



360

Compound **360** was prepared starting from compound **372** (200 mg, 1.19 mmol) according to the above procedure in 39% yield (102 mg, 0.46 mmol).

HPLC t_R = 14.0 min (method A).

^{1}H NMR (500 MHz, DMSO-d_6): δ 10.09 (d, *J* = 2 Hz , 1H), 8.99 (s, 1H), 8.41 (s, 1H), 7.89 (d, *J* = 9 Hz, 1H), 7.84 (d, *J* = 7.5 Hz, 2H), 7.53 (dd, *J* = 7.5, 7.5 Hz, 2H), 7.44 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.31 (dd, *J* = 2, 9 Hz, 1H), 7.23 (d, *J* = 2 Hz , 1H).

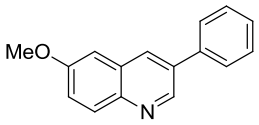
^{13}C NMR (125 MHz, DMSO-d_6): δ 155.9, 146.1, 142.2, 137.4, 132.8, 131.1, 130.1, 129.2, 128.0, 127.1, 122.0, 108.7.

FTIR (KBr) ν_{max} cm^{-1}: 2952, 1620, 1496, 1457, 1376, 1246, 1217, 1166, 950, 898, 763, 701.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 206, 255, 342.

HRMS-EI *m/z*: measured 221.0838 ([M] $^+$, calcd. 221.0841 for $C_{15}H_{11}NO$). MS (EI) *m/z* (% relative int.): 221 [M^+] (100), 192 (7), 165 (11), 139 (5).

6-Methoxy-3-phenylquinoline (361)



361

Compound **361** was prepared starting from compound **373** (178 mg, 0.98 mmol) according to the above procedure in 53% yield (123 mg, 0.52 mmol). Analytical data matched with the published values (Monrad and Madsen, 2011).

HPLC t_R = 18.6 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.03 (s, 1H), 8.21 (s, 1H), 8.04 (d, *J* = 9 Hz, 1H), 7.71 (d, *J* = 7.5 Hz, 2H), 7.53 (dd, *J* =7.5, 7.5 Hz, 2H), 7.44 (dd, 7.5, 7.5 Hz, 1H), 7.38 (dd, 7.5, 1.5 Hz, 1H), 7.14 (s, 1H), 3.96 (s, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 158.3, 147.6, 143.7, 138.3, 134.3, 132.3, 130.8, 129.3, 129.2, 128.2, 127.6, 122.4, 105.5, 55.8.

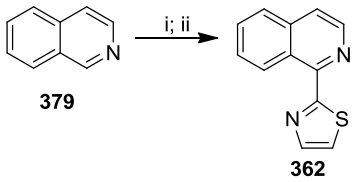
FTIR (KBr) ν_{max} cm^{-1}: 3002, 2834, 1620, 1496, 1463, 1374, 1351, 1244, 1213, 1164, 1029, 902, 831, 763, 697.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 207, 255, 340.

HRMS-EI *m/z*: measured 235.1000 ([M] $^+$, calcd. 235.0997 for $C_{16}H_{13}NO$). MS (EI) *m*/*z* (% relative int.): 235 [M^+] (100), 205 (7), 193 (10), 192 (63), 165 (15), 139 (6), 115 (5).

4.4.2 Synthesis of isoquinoline derivatives

4.4.2.1 1-(2-Thiazolyl)isoquinoline (362)



Reagents and conditions: (i) $ClCO_2Et$, DCM, 2-(trimethylsilyl)thiazole, r.t., 54%; (ii) *o*-chloranil, C_6H_6, reflux, 70%.

To a solution of isoquinoline (**379**, 100 mg, 0.77 mmol) in DCM was added ethylchloroformate (73 mg, 0.77 mmol) at 0 ºC and the reaction mixture was stirred for 30 min. Next, 2-(trimethylsilyl)thiazole (122 mg, 0.77 mmol) was added and the reaction mixture was stirred at r.t. for 3 h. Concentration of the reaction mixture followed by FCC separation (SiO_2, DCM/Hex) of the residue afforded 1-(2-thiazolyl)-1(2H)-isoquinolinecarboxylic acid ethyl ester **380** (113 mg, 0.39 mmol) as a pale yellow solid in 54% yield. To a solution of compound **380** (110 mg, 0.404 mmol) in benzene (5 ml) *o*-chloranil (108 mg, 0.44 mmol) was added and the reaction mixture was refluxed for 5 h. The reaction mixture was diluted with 5% NaOH (10 ml) and extracted with DCM (2 × 30 ml). Combined organic extract was dried over Na_2SO_4 and

concentrated to yield the crude which was separated by FCC (SiO_2, DCM/Hex) to afford the product **362** (60 mg, 0.28 mmol) as a white solid in 70% yield (Dondoni, Dall' Occo et al., 1984).

HPLC t_R = 14.7 ± 0.5 min (method A).

^{1}H NMR (500 MHz, CDCl3): δ 9.50 (m, 1H), 8.57 (d, *J* = 5.5 Hz, 1H), 8.07 (d, *J* = 3 Hz, 1H), 7.88 (m, 1H), 7.74 (m, 3H), 7.52 (d, *J* = 3 Hz, 1H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 1.0, 149.7, 144.2, 141.9, 137.5, 130.6, 128.8, 128.0, 127.1, 125.7, 122.4, 122.2.

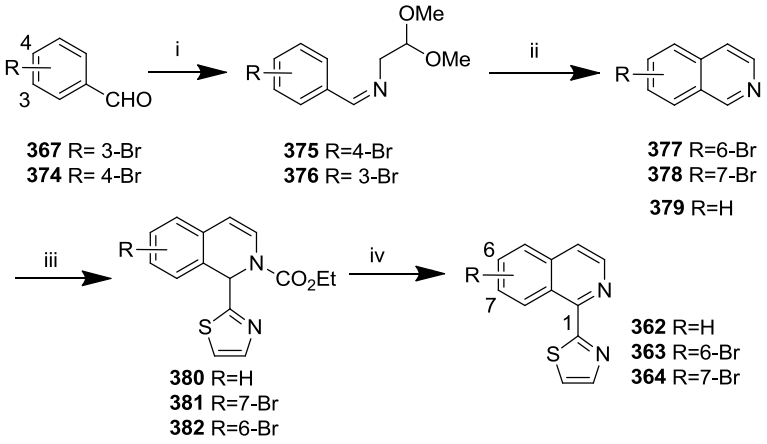
FTIR (KBr) ν_{max} cm^{-1}: 3050, 1579, 1550, 1496, 1425, 1350, 1312, 1270, 1236, 1097, 946, 878, 828, 747.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 230, 295, 343.

HRMS-EI *m/z*: measured 212.0413 ([M] $^+$, calcd. 212.0408 for $C_{12}H_8N_2S$). MS (EI) *m/z* (% relative int.): 212 [M^+] (100), 186 (10), 155 (9), 128 (14), 101 (11).

4.4.2.2 Synthesis of compounds 363 and 364

General procedure



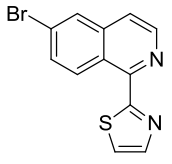
Reagents and conditions: (i) Aminoacetaldehyde dimethyl acetal, toluene, reflux (Dean-Stark); (ii) P_2O_5, H_2SO_4, 160 °C, 15-20% (Czako, Kurti et al., 2009); (iii) $ClCO_2Et$, DCM, 2-trimethylsilylthiazole, r.t.; (iv) *o*-chloranil, toluene, reflux, 15-32%.

Aminoacetaldehyde dimethyl acetal (3.0 eq) was added to a solution of bromobenzaldehydes **367** and **374** (1 eq) in toluene (30 ml) and refluxed (using a Dean-

Stark apparatus) at 120 ºC. After consumption of the starting material (2 h), the reaction mixture was evaporated under reduced pressure to afford the crude acetals **375** and **376**. A cooled viscous solution of the acetals **375** and **376** in conc. H_2SO_4 (2 ml) was added to a cooled solution of P_2O_5 in conc. H_2SO_4 (0.5 ml) and the reaction mixture was heated to 160 ºC. After 30 min, reaction mixture was cooled to r.t. and neutralized with 10 N NaOH and extracted in EtOAc (3 × 40 ml). Combined organic extract was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford the crude product, which was purified by FCC to yield 6-bromoisoquinoline (**377**, 30 mg, 0.14 mmol) in 14% yield and 7-bromoisoquinoline (**378,** 99 mg**,** 0.47 mmol) in 22% yield (Jiang, Duckett et al., 2007).

Ethylchloroformate (1 eq) was added to a solution of isoquinolines **377-379** (1 eq) in DCM at 0 ºC and the reaction mixture was stirred for 30 min. Next, 2-(trimethylsilyl)thiazole (1 eq) was added and the reaction mixture was stirred at r.t. for 3 h. Concentration of the reaction mixture followed by FCC separation (SiO_2, DCM/Hex) of the residue afforded 1-(2-thiazolyl)-1(2H)-isoquinolinecarboxylic acid ethyl esters **380-382** (113 mg, 0.39 mmol). Further, solution of compounds **380-382** (1 eq) in benzene (5 ml) *o*-chloranil (1 eq) was added and the reaction mixture was refluxed for 5 h. The reaction mixture was diluted with 5% NaOH (10 ml) and extracted with DCM (2 × 30 ml). Combined organic extract was dried over Na_2SO_4 and concentrated to yield the crude which was separated by FCC (SiO_2, DCM/Hex) to afford the products **362-364** (Dondoni, Dall' Occo et al., 1984).

6-Bromo-1-(2-thiazolyl)isoquinoline (363)



363

6-Bromoisoquinoline (**377**, 30 mg, 0.14 mmol) was synthesized starting from 4-bromobenzaldehyde (**374**, 200 mg, 1.08 mmol) in 14% yield. Further, compound **363**

was synthesized starting from 6-bromoisoquinoline (**377**, 100 mg, 0.48 mmol) in 15% yield over two steps (21 mg**,** 0.07 mmol).

HPLC t_R = 22.4 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.77 (d, *J* = 9 Hz, 1H), 8.58 (d, *J* = 5.5 Hz, 1H), 8.06 (m, 2H), 7.79 (dd, *J* = 2, 9 Hz, 1H), 7.61 (d, *J* = 5.5 Hz, 1H), 7.53 (d, *J* = 3 Hz, 1H).

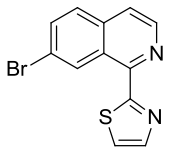
^{13}C NMR (125 MHz, $CDCl_3$): δ 0.6, 149.9, 144.4, 143.0, 138.7, 132.3, 130.1, 129.1, 125.7, 124.0, 122.5, 121.2.

FTIR (KBr) ν_{max} cm^{-1}: 2924, 2852, 1782, 1608, 1546, 1426, 1246, 1183, 947, 880, 810.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 240, 345.

HRMS-EI *m/z*: measured 291.9494 ($[M]^+$, calcd. 291.9493 [100%] for $C_{12}H_7N_2S^{81}Br$), 289.9519 ($[M]^+$, calcd. 289.9513 for $C_{12}H_7N_2S^{81}Br$). MS (EI) *m/z* (% relative int.): 292 [M^+] (100), 290 (95), 264 (7), 248 (53), 211 (22), 153 (9), 127 (12), 100 (34), 58 (15).

7-Bromo-1-(2-thiazolyl)isoquinoline (364)



364

7-Bromoisoquinoline (**378,** 99 mg**,** 0.47 mmol) was synthesized starting from compound **367** in 22% yield (Jiang, Duckett et al., 2007). Further, compound **364** was synthesized starting from 7-bromoisoquinoline **(378**, 100 mg, 0.48 mmol) in 24% yield over two steps (35 mg**,** 0.12 mmol).

HPLC t_R = 21.0 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 10.12 (s, 1H), 8.58 (d, *J* = 5.5 Hz, 1H), 8.08 (d, *J* = 3 Hz, 1H), 7.80 (dd, *J* = 9, 2 Hz, 1H), 7.73 (d, *J* = 9 Hz, 1H), 7.67 (d, *J* = 5.5 Hz, 1H), 7.53 (d, *J* = 3 Hz, 1H).

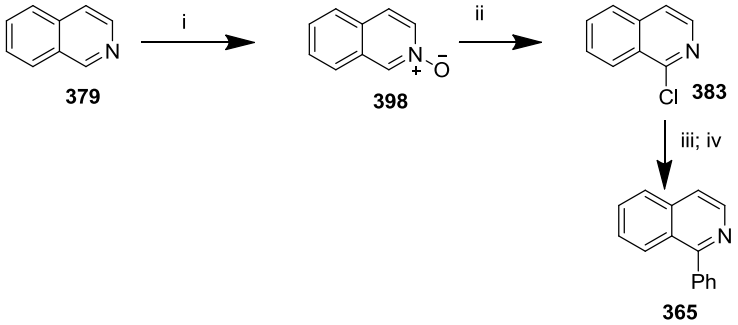
^{13}C NMR (125 MHz, $CDCl_3$): δ 0.3, 148.6, 144.5, 142.3, 136.0, 134.2, 130.5, 128.6, 126.3, 123.1, 122.5, 122.1.

FTIR (KBr) ν_{max} cm^{-1}: 3089, 1781, 1573, 1544, 1491, 1425, 1247, 1103, 947, 850, 813, 732.

UV (HPLC, $CH_3OH–H_2O$) λ_{max} (nm): 240, 302, 350.

HRMS-EI *m/z*: measured 291.9483 ($[M]^+$, calcd. 291.9493 [100%] for $C_{12}H_7N_2S^{81}Br$), 289.9512 ($[M]^+$, calcd. 289.9513 for $C_{12}H_7N_2SBr$). MS (EI) *m/z* (% relative int.): 292 (100), 290 [M^+] (99), 264 (6), 211 (32), 185 (5), 162 (15), 153 (15), 127 (13), 100 (24), 58 (15).

4.4.2.3 1-Phenylisoquinoline (365)



Reagents and conditions: (i) H_2O_2, AcOH, 80 °C, (Barber, Dickinson et al., 2004); (ii) $POCl_3$, $CHCl_3$, 80 °C (Alcock, Wirth et al., 1993); (iii) I_2, THF; (iv) PhMgCl, Fe, THF.

A solution of H_2O_2 (2.5 ml) and acetic acid (5 ml) was heated to 80 ºC. After 1 hr, isoquinoline (**379**, 200 mg, 1.55 mmol) was added to the reaction mixture and heated at the same temperature for 16 h. The reaction mixture was diluted with water (10 ml) and concentrated using a rotary evaporator to 1/3rd volume; extracted with EtOAc (3 × 30 ml). Combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure to afford the crude isoquinoline-*N*-oxide (**398**) (Barber, Dickinson et al., 2004). $POCl_3$ (119 µl, 1.27 mmol) was added dropwise to a solution of *N*-oxide **CP064** (88 mg, 0.60 mmol) in $CHCl_3$ and refluxed at 80 ºC. After 3 hrs, reaction mixture was cooled to r.t., and diluted with ice cold water (10 ml), basified with 33% NH_4OH (1 ml) and extracted with EtOAc (3 × 30 ml). The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure to afford 1-chloroisoquinoline (**383**, 38 mg, 0.23 mmol) in 42% yield (Alcock, Wirth et al., 1993). Iodine (18 mg, 0.073 mmol) was added to a solution of compound **383** (12 mg, 0.073 mmol) in THF (2 ml) and stirred at r.t. After 30 min, Fe powder (16 mg, 0.294 mmol) followed by PhMgCl

(2 M in THF, 43 µl, 0.087 mmol) were added and stirred at r.t. for 6 h. After complete consumption of the starting material by TLC, the reaction mixture was diluted with ice-cold water (20 ml) and extracted EtOAc (3 × 30 ml). The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure to afford 1-phenylisoquinoline (**365**, 10 mg, 0.05 mmol) in 67% yield. Analytical data matched with the published values (Larivee, Mousseau et al., 2008).

HPLC t_R = 16.2 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.63 (d, *J* = 5.5 Hz, 1H), 8.12 (d, *J* = 8.5 Hz, 1H), 7.90 (d, *J* = 8.5 Hz, 1H), 7.71 (m, 3H), 7.67 (d, *J* = 5.5 Hz, 1H), 7.51 (m, 4H).

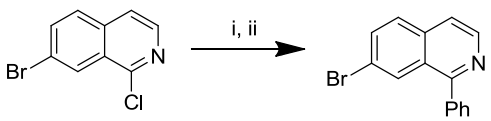
^{13}C NMR (125 MHz, $CDCl_3$): δ 161.0, 142.5, 139.9, 137.1, 130.2, 130.1, 128.8, 128.6, 127.8, 127.4, 127.2, 127.0, 1201.1.

FTIR (KBr) ν_{max} cm^{-1}: 3053, 1695, 1556, 1444, 1385, 973, 825, 763, 699.

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 229, 276, 324.

HRMS-EI *m/z*: measured 205.0880 ([M] $^+$, calcd. 205.0891 for $C_{15}H_{11}N$). MS (EI) *m/z* (% relative int.): 205 [M^+] (48), 204 (100), 176 (6), 102 (9), 69 (6).

4.4.2.4 7-Bromo-1-phenylisoquinoline (366)



Reagents and conditions: (i) I_2, THF; (ii) PhMgCl, Fe, THF.

Repetition of the above procedure starting from compound **384** (40 mg, 0.16 mmol) instead of compound **383** yielded compound **366** (21 mg, 0.07 mmol) in 47% yield.

HPLC t_R = 23.4 min (method A).

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.64 (d, *J* = 6 Hz, 1H), 8.07 (d, *J* = 2 Hz, 1H), 7.99 (d, *J* = 9 Hz, 1H), 7.68 (dd, *J* = 2, 7.5 Hz, 2H), 7.62 (dd, *J* = 2, 9 Hz, 1H), 7.58-7.53 (m, 4H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 161.2, 143.6, 139.3, 138.2, 130.9, 130.1, 129.7, 129.3, 129.1, 128.7, 125.3, 125.1, 119.0.

FTIR (KBr) ν_{max} cm^{-1}: 3061, 3033, 2923, 1480, 1429, 1261, 1091, 1042, 1008, 903, 800, 728, 697.

UV (HPLC, $CH_3OH–H_2O$) λ_{max} (nm): 220, 258, 328.

HRMS-EI *m/z*: measured 284.9969 ($[M]^+$, calcd. 284.9976 [10%] for $C_{15}H_{10}N^{81}Br$), 282.9985 ($[M]^+$, calcd. 282.9997 for $C_{15}H_{10}NBr$). MS (EI) *m/z* (% relative int.): 285 (10), 283 [M^+] (10), 237 (26), 204 (13), 184 (41), 149 (19), 108 (6), 102 (5), 98 (26), 91 (100), 84 (27), 65 (25).

4.5 Antifungal activity

The antifungal activity of compounds was determined using a mycelial radial growth bioassay. Isolates of *L. maculans* (isolates BJ-125/ UAMH-9410) were grown on V_8 agar plates for 14 days at 23ºC under constant light. Sterile tissue culture plates (6-well, 33 mm diameter) were used in all bioassays. Solutions of each compound in ACN / DMSO were added to PDA (1% ACN / DMSO in final volume) to prepare the plates (0.50, 0.20 and 0.10 mM). Control plates were prepared to contain 1% ACN / DMSO in PDA. Plates containing test and control agar (2 ml per well) were inoculated with mycelium plugs [4 mm, cut from 7-day-old V_8 plates of *L. maculans* (isolates BJ-125/ UAMH-9410] placed upside down on the center of each plate and incubated under constant light for 5 days (Pedras and Jha, 2006). Mycelial growth in each well was measured and % inhibition values were calculated as previously reported (Pedras and Jha, 2006). All bioassay experiments were carried out in triplicate, at least two times.

4.6 Metabolism studies

Metabolism studies of compounds in fungal cultures were carried out as previously reported (Pedras and Suchy, 2006). Erlenmeyer flasks (250 ml) each containing 100 ml minimal media were employed. Media was inoculated with spores of *L. maculans* (isolate BJ-125, 10^6/ml) and incubated at 23 ºC on a shaker at 120 rpm under constant light. After two days, phytoalexins, related compounds or potential inhibitors of brassinin detoxification (in ACN/DMSO, final conc 0.10 mM) were added to the

cultures as well as uninoculated media (control). Samples (2 ml or 5 ml) were withdrawn immediately and at intervals between 6-72 h after incubation; and were either frozen or immediately extracted with EtOAc (2 × 5 ml). The organic extracts were concentrated; dissolved in ACN/MeOH (1 ml) and analyzed by HPLC.

4.7 Screening of potential inhibitors of brassinin detoxification

Erlenmeyer flasks (125 ml) each containing 50 ml minimal media were employed. Media was inoculated with spores of *L. maculans* (isolate BJ-125, 10^4/ml) and incubated at 23 ºC on a shaker at 120 rpm under constant light. After five days, mycelia were filtered, resuspended in water (50 ml) and further incubated under same conditions. After 72 h, potential inhibitors (final concentration 0.10 mM) in ACN/DMSO, followed by brassinin (**17**, 0.10 mM) in ACN were added to three flasks (triplicates). Only brassinin (**17**, 0.10 mM) in ACN was added to three flasks (control). Samples (2 ml) were withdrawn immediately and after 6, 15, 24, 48 and 72 h of incubation; and were either frozen or immediately extracted with EtOAc (2 × 5 ml). The organic extracts were concentrated, dissolved in ACN (1 ml) and analyzed by HPLC.

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Appendix

A1A Structures of cruciferous phytoalexins

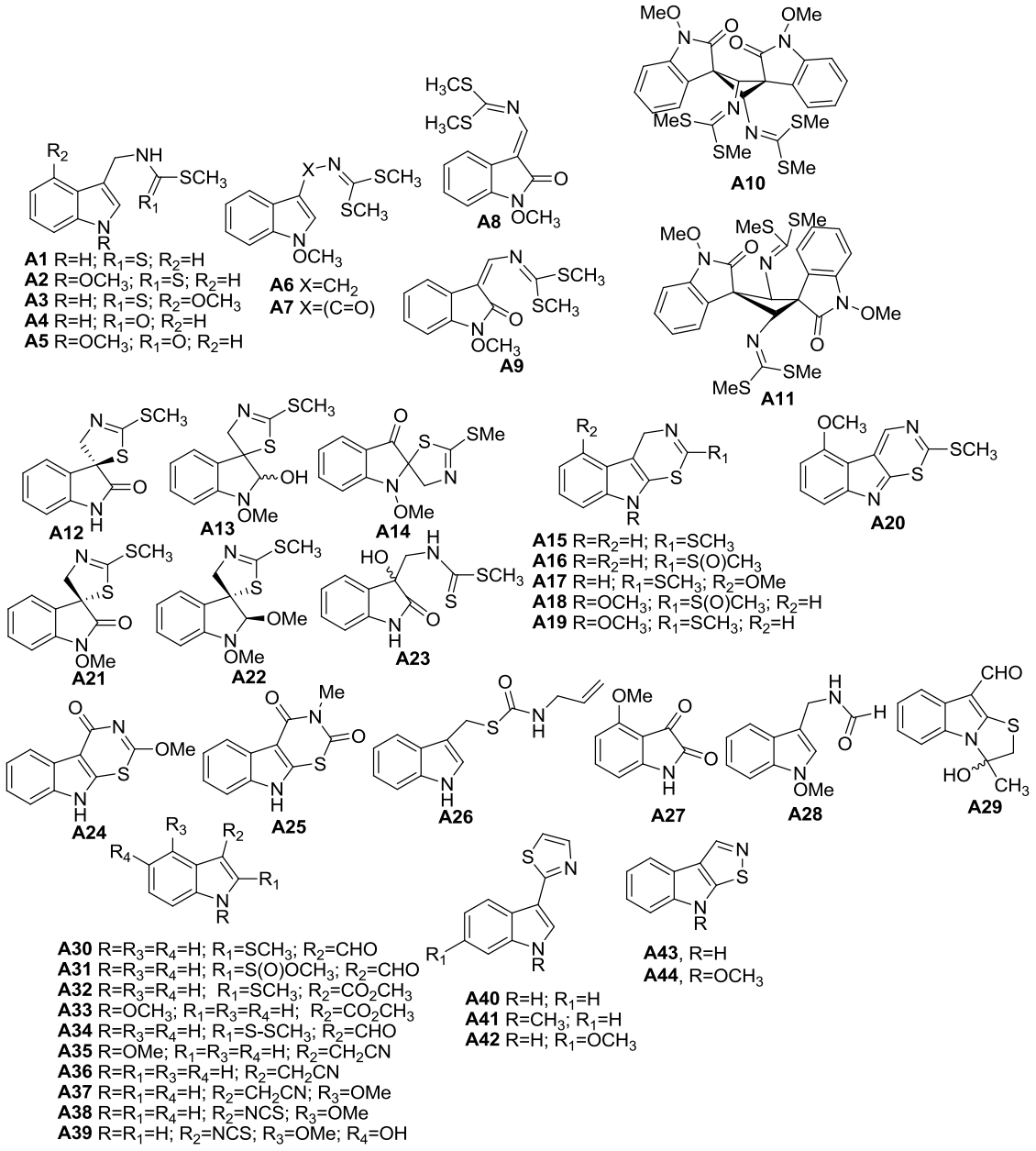


Figure A. 1 Phytoalexins from Brassicaceae: brassinin (**A1**); 1-methoxybrassinin (**A2**); 4-methoxybrassinin (**A3**); brassitin (**A4**);1-methoxybrassitin (**A5**); 1-methoxybrassenin A (**A6**); 1-methoxybrassenin B (**A7**); wasalexins, A (**A8**) and B (**A9**); biswasalexins, A (**A10**) and B (**A11**); spirobrassinin (**A12**); 1-methoxyspirobrassinin (**A13**); 1-methoxyspirobrassinol (**A14**); 1-methoxyspirobrassinol methyl ether (**A15**); erucalexin

(**A16**); dioxibrassinin (**A17**); cyclobrassinin (**A18**); cyclobrassinin sulfoxide (**A19**); 4-methoxycyclobrassinin (**A20**); sinalbin A (**A21**); sinalbin B (**A22**); dehydro-4-methoxycyclobrassinin (**A23**); cyclobrassinone (**A24**); rutalexin (**A25**); brussalexin (**A26**); isalexin (**A27**); caulilexin B (**A28**); brassicanal B (**A29**); brassicanal A (**A30**); brassicanal C (**A31**); brassicanate A (**A32**); methyl 1-methoxyindole-3-carboxylate (**A33**); caulilexin A (**A34**); caulilexin C (**A35**); indolyl-3-acetonitrile (**A36**) arvelexin (**A37**); rapalexins, A (**A38**) and B (**A39**); camalexin (**A40**); 1-methylcamalexin (**A41**); 6-methoxycamalexin (**A42**); brassilexin (**A43**); sinalexin (**A44**) (Pedras, Yaya et al., 2011b).

A1B Structures of phytoalexin-related compounds not metabolized by pathogenic fungi

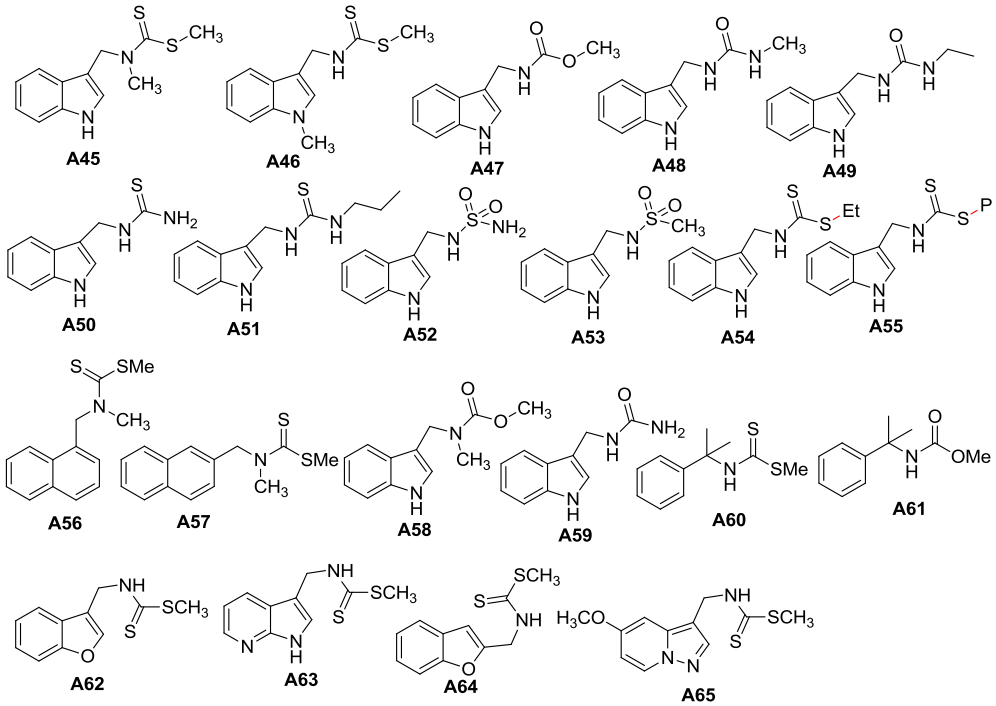


Figure A. 2 Structures of brassinin analogs resistant to fungal metabolism: compounds not metabolized by *Leptosphaeria maculans* (virulent on canola) **A45**-**A61** (Pedras and Jha, 2006; Pedras, Jha et al., 2007b); compounds not metabolized by *Leptosphaeria biglobosa* (virulent on mustard) **A45**-**A57** (Pedras, Gadagi et al., 2007a; Pedras, Minic et al., 2009b); compounds not metabolized by *Sclerotinia sclerotiorum* **A62**-**A65** (Pedras and Hossain, 2007).

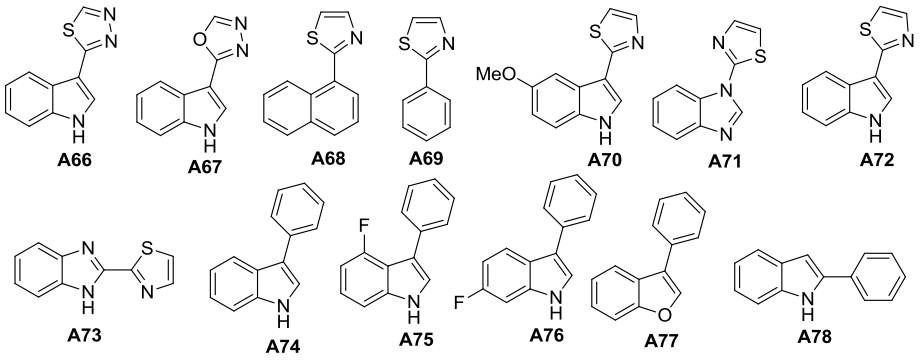


Figure A. 3 Structures of camalexin analogs resistant to fungal metabolism: compounds not metabolized by *Leptosphaeria maculans* (virulent on canola) **A66**-**A73** (Pedras, Minic et al., 2009c); compounds not metabolized by *Leptosphaeria biglobosa* (virulent on mustard) **A72** and **A73** (Pedras, Gadagi et al., 2007a); compounds not metabolized by *Sclerotinia sclerotiorum* **A73**-**A78** (Pedras and Hossain, 2007).

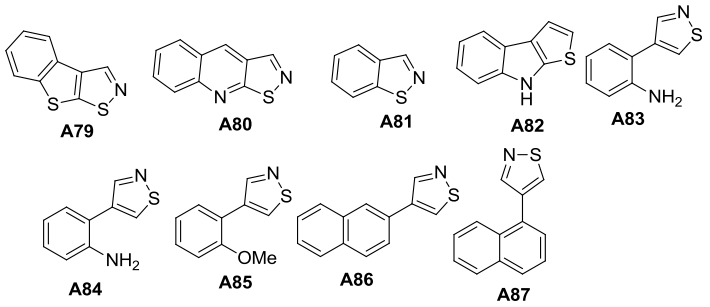
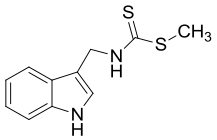
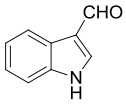
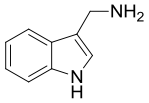
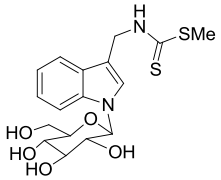
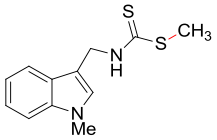
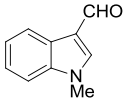
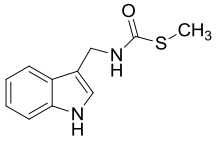
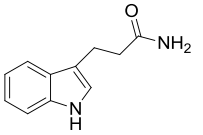
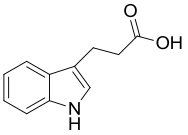
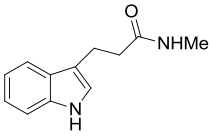
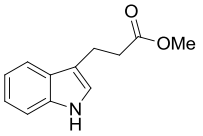
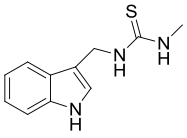
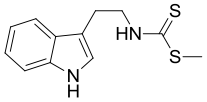
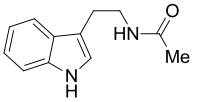
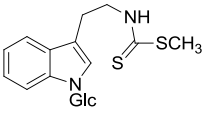
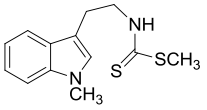
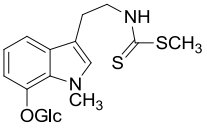


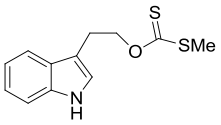
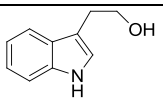
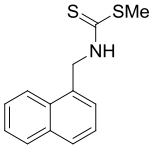
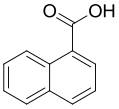
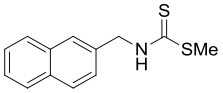
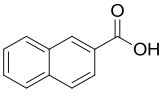
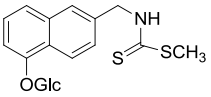
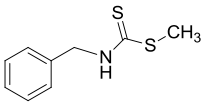
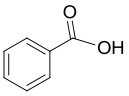
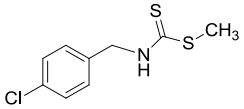
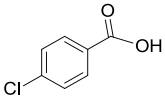
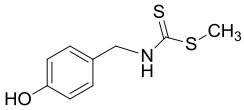
Figure A. 4 Structures of brassilexin analogs resistant to fungal metabolism by *Leptosphaeria maculans* (virulent on canola) **A79**-**A87** (Pedras and Suchy, 2006).

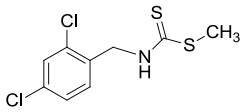
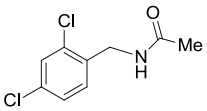
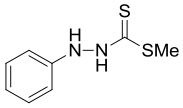
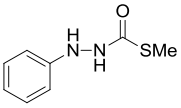
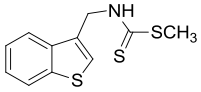
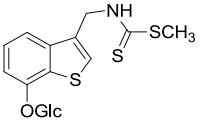
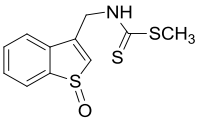
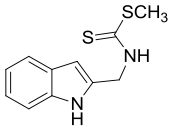
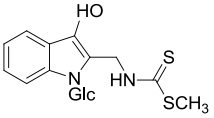
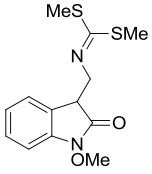
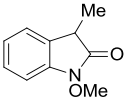
A1C Summary of metabolism of phytoalexins and analogs

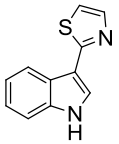
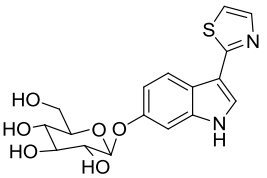
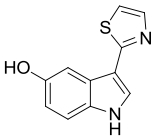
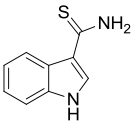
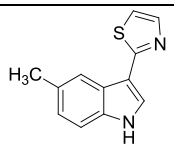
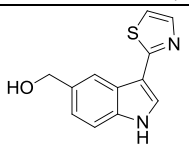
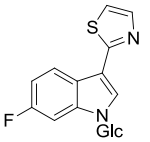
Table A. 1 Antifungal activity of phytoalexin analogs and their biotransformation products in cruciferous fungal pathogens *L. m* [*Leptosphaeria maculans* (virulent on canola)], *L. m*L2 [*L. maculans* (virulent on mustard)], *L. b* (*Leptosphaeria biglobosa*); *S. s* (*Sclerotinia sclerotiorum*), *R. s* (*Rhizoctinia solani*), *A. b* (*Alternaria brassicicola*) and *B. c* (*Botrytis cineria*). c.i. = complete inhibition; n.i. = no inhibition.

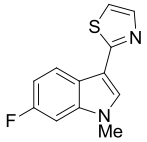
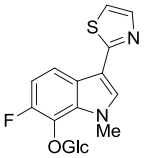
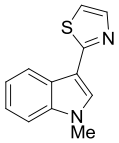
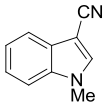
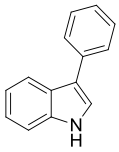
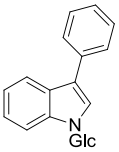
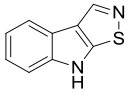
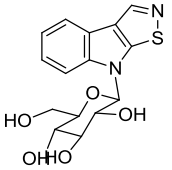
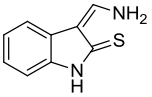
Compound/ % growth inhibition at 0.50 mM (Fungal species)	Product / Fungal species (Ref)
 Brassinin/ 55% (*L. m*); 38% (*L. m* L2); 39% (*A. b*); 100% (*B. c*); c.i. (*S. s*).	 Indolyl-3-carboxaldehyde/ *L. m* (Pedras and Taylor, 1993);  Indolyl-3-methanamine/*L. m*L2 (Pedras, Gadagi et al., 2007a); *A. b* (Pedras, Chumala et al., 2009a); *B. c* (Pedras, Hossain et al., 2011a);  1-β-D-glucopyranosylbrassinin/ *S. s* (Pedras, Ahiahonu et al., 2004a)
 1-Methylbrassinin	 1-Methyl-indolyl-3-carboxaldehyde/*L. m* (Pedras, Jha et al., 2007b).
 Brassitin	Indolyl-3-carboxaldehyde/*L. m* (Pedras, Jha et al., 2007b)

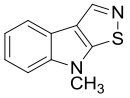
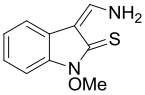
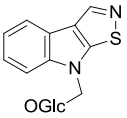
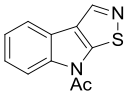
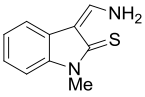
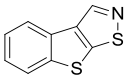
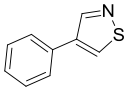
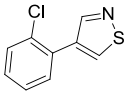
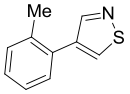
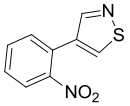
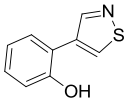
Compound/ % growth inhibition at 0.50 mM (Fungal species)	Product / Fungal species (Ref)
 Indolyl-3-propanamide/ 48% (*L. m*).	 Indolyl-3-propionic acid/ *L. m* (Pedras and Jha, 2006).
 Methyl indolyl-3-propanamide/ n.i. (*L. m*[a]).	Indolyl-3-propionic acid/ *L. m* (Pedras and Jha, 2006).
 Methyl indolyl-3-propionate/ 48% (*L. m*[a]); n.i. (*L. m*[b]).	Indolyl-3-propionic acid/ *L. m* (Pedras and Jha, 2006); *L. m*L2 (Pedras, Gadagi et al., 2007a).
 *N*-(Indol-3-ylmethyl)-*N′*-methylthiourea/ 73% (*L. m*).	Indolyl-3-carboxaldehyde/*L. m* (Pedras and Jha, 2006).
 Methyl tryptaminedithiocarbamate/ c.i. (*L. m*); 95% (*L. m*L2); c.i. (*S. s*).	 N_b-Acetyltryptamine/ *L. m* (Pedras and Okanga, 2000); *L. m*L2 (Pedras, Gadagi et al., 2007a; Pedras and Okanga 2000).  Methyl 1-(β-D-glucopyranosyl)tryptaminedithiocarbamate/ *S. s* (Pedras, Ahiahonu et al., 2004a)
 Methyl 1-methyltryptamine dithiocarbamate/ c.i. (*S. s*).	 Methyl 7-(*O*-β-D-glucopyranosyl)-1-methyltryptaminedithiocarbamate/ *S. s* (Pedras, Ahiahonu et al., 2004a; Pearson., Ph.D. thesis, 2004).

Compound/ % growth inhibition at 0.50 mM (Fungal species)	Product / Fungal species (Ref)
 Tryptophol dithiocarbonate/ 41% (*L. m*); 41% (*L. m*L2);	 Tryptophol/ *L. m* [a] (Pedras and Jha, 2006); *L. m* [b] (Pedras, Gadagi et al., 2007a)
 Methyl *N*-(1-naphthalenylmethyl)dithiocarbamate / 44% (*L. m*)/ 80% (*S. s*).	 1-Naphthoic acid/ *L. m* (Pedras and Jha, 2006); Undetermined products/ *S. s* (Pedras, Ahiahonu et al., 2004a).
 Methyl *N*-(2-naphthalenylmethyl)dithiocarbamate / 40% (*L. m*); 80% (*S. s*).	 2-Naphthoic acid/ *L. m* (Pedras and Jha, 2006).  Methyl 6-oxy-(*O*-β-D-glucopyranosyl)-*N*-(2-naphthalenylmethyl)dithiocarbamate/*S. s* (Pedras, Ahiahonu et al., 2004a).
 Methyl *N*-benzyldithiocarbamate/ c.i. (*L. m*).	 Benzoic acid/ *L. m* (Pedras and Jha, 2006).
 Methyl *N*-(4-chlorobenzyl)dithiocarbamate.	 4-Chlorobenzoic acid/*L. m* (Pedras, Khan et al., 1997); *L. b* (Pedras, Khan et al., 1997).
 Methyl *N*-(4-hydroxybenzyl)dithiocarbamate.	Undetermined polar metabolites/ *L. m* (Pedras, Khan et al., 1997); *L. b* (Pedras, Khan et al., 1997).

Compound/ % growth inhibition at 0.50 mM (Fungal species)	Product / Fungal species (Ref)
 Methyl *N*-(2,4-dichlorobenzyl)dithiocarbamate.	 *N*-(2,4-dichlorobenzyl)acetamide/ *L. m* (Pedras, Khan et al., 1997); *L. b* (Pedras, Khan et al., 1997).
 Methyl phenyldithiocarbazate/ c.i. (*L. m*).	 *S*-methyl-3-phenylthiocarbazate /*L. m* (Pedras and Jha, 2006).
 Methy (thianaphthen-3-yl)methyldithiocarbamate/ 89% (*S. s*).	 Methyl (7-oxy-*O*-β-D-glucopyranosylthianaphthen-3-yl)methyl-dithiocarbamate/*S. s* (Pedras and Hossain, 2007).  Methyl (thianaphthen-3-yl-1-S-oxide)methyldithiocarbamate/*S. s* (Pedras and Hossain, 2007).
 Isobrassinin/ c.i. at 0.30 mM (*S. s*).	 Methyl (1-β-D-glucopyranosyl-3-hydroxylindol-2-yl)methyldi-thiocarbamate/ *S. s* (Pedras and Hossain, 2007).
 Dihydrowasalexin/ 63% (*L. m*); 34% (*L. m*L2).	 1-Methoxy-3-methylindol-2-one/ *L. m* (Pedras and Suchý, 2006); *L. m*L2 (Pedras and Suchý, 2006).

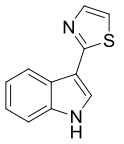
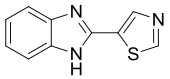
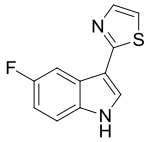
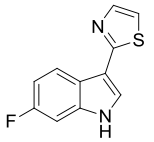
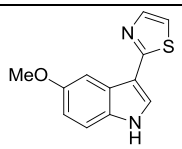
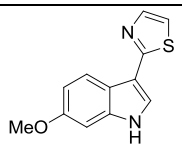
Compound/ % growth inhibition at 0.50 mM (Fungal species)	Product / Fungal species (Ref)
 Camalexin/ c.i. (*L. m*, *L. b*, *L. m*L2, *A. b*, *S. s*, *R. s*, *B. c*)	No metabolism/ *L. m* (Pedras, Khan et al., 1998); No metabolism/ *L. b* (Pedras, Khan et al., 1998); No metabolism/ *L. m*L2 (Pedras, Gadagi et al., 2007a); No metabolism/ *A. brassicae* (Pedras, Khan et al., 1998);  6-oxy-(*O*-β-D-glucopyranosyl)camalexin/ *S. s* (Pedras and Ahiahonu, 2002)  5-Hydroxycamalexin/ *R. s* (Pedras and Khan, 1997)  Indolyl-3-thiocarboxamide/ *B. c* (Pedras, Hossain et al., 2011a).
 5-Methylcamalexin/ c.i. at 0.25 mM (*R.* s).	 5-Hydroxymethylcamalexin/ *R.* s (Pedras and Liu, 2004).
 6-Fluorocamalexin/ 70% (*S. s*).	 6-Fluoro-(1-β-D-glucopyranosyl)brassinin/ *S. s* (Pedras and Ahiahonu, 2002).

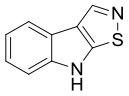
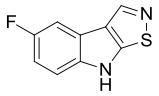
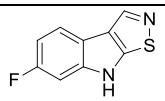
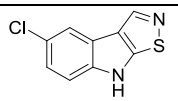
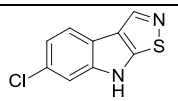
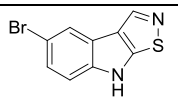
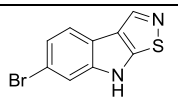
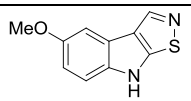
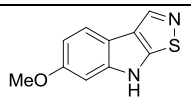
Compound/ % growth inhibition at 0.50 mM (Fungal species)	Product / Fungal species (Ref)
 6-Fluoro-1-methylcamalexin/ c.i. (*S. s*).	 6-Fluoro-1-methyl-7-(*O*-β-d-glucopyranosyl)camalexin/ *S. s* (Pedras and Ahiahonu, 2002).
 1-Methylcamalexin/ c.i. at 0.25 mM (*R.* s).	 1-Methylindolyl-3-carbonitrile/ *R.* s (Pedras and Liu, 2004).
 3-Phenylindole/ c.i. at 0.10 mM (*S.* s); C.I. (*L. m* [a]).	 1-β-D-glucopyranosyl-3-phenylindole/ *S.* s (Pedras and Hossain, 2007).
 Brassilexin/ c.i. [*L. m, L. m*L2, *S. s, B. c*]	 1-β-D-glucopyranosylbrassilexin/ *S. s* (Pedras and Hossain, 2006);  3-Aminomethyleneindole-2-thione/ *L. m* (Pedras and Suchy, 2005); *L. m*L2 (Pedras and Snitynsky, 2010); *B. c* (Pedras, Hossain et al., 2011a).

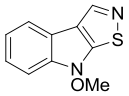
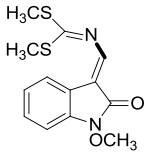
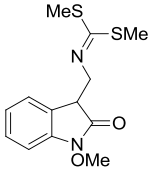
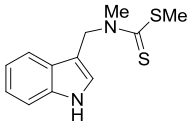
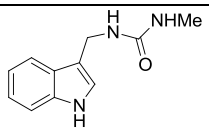
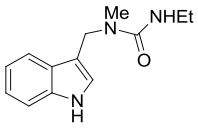
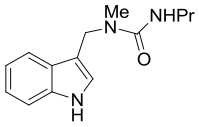
Compound/ % growth inhibition at 0.50 mM (Fungal species)	Product / Fungal species (Ref)
 1-Methylbrassilexin/ c.i. (*L. m*); c.i. at 0.30 mM (*S. s*).	 3-Aminomethylene-1-methoxyindole-2-thione/ *L. m* (Pedras and Suchy, 2005).  1-Methyl-(oxy-O-β-D-glucopyranosyl)brassilexin/ *S.* s (Pedras and Hossain, 2006).
 1-Acetylbrassilexin	 3-Aminomethylene-1-methylindole-2-thione/ *L. m* (Pedras and Suchy, 2005).
 Isothiazolo[5,4-*b*]thianaphthene/ c.i. (*L. m*)	Undetermined product/ *L. m* (Pedras and Suchy, 2006).
 4-Phenylisothiazole/ 69% (*L. m*)	Undetermined product/ *L. m* (Pedras and Suchy, 2006).
 4-(2-Chlorophenyl)isothiazole/ 79% (*L. m*)	Undetermined product/ *L. m* (Pedras and Suchy, 2006).
 4-(2-Tolyl)isothiazole/ 65% (*L. m*)	Undetermined product/ *L. m* (Pedras and Suchy, 2006).
 4-(2-Nitrophenyl)isothiazole/ 67% (*L. m*)	Undetermined product/ *L. m* (Pedras and Suchy, 2006).
 4-(2-Hydroxyphenyl)isothiazole/ c.i. (*L. m*)	Undetermined product/ *L. m* (Pedras and Suchy, 2006).

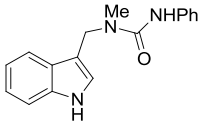
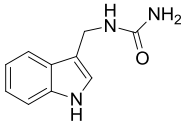
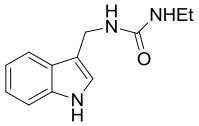
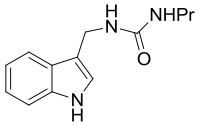
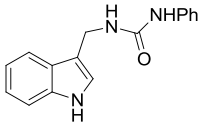
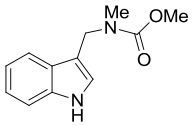
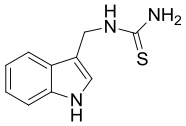
A1D Summary of inhibition of BOLm

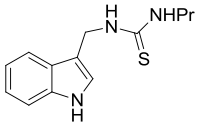
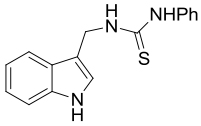
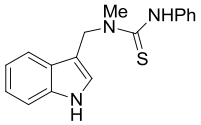
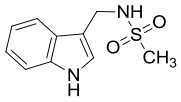
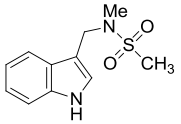
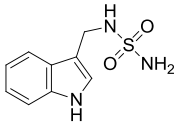
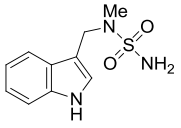
Table A. 2 Inhibition of BOLm and antifungal activities against *Leptosphaeria maculans* of tested compounds (Pedras et al., 2009; Pedras et al., 2010). c.i. = complete inhibition; n.i. = no inhibition.

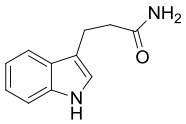
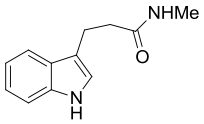
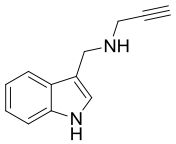
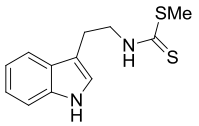
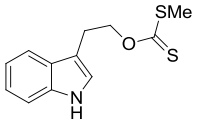
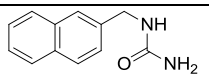
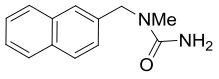
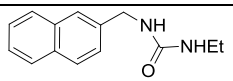
Compound	% Inhibition of BOLm		Antifungal activity at 0.10 mM/ Ref
	0.10 mM	0.30 mM	
 Camalexin	30 ± 4	53 ± 4	35 ± 5/ (Pedras, Minic et al., 2009c).
 Thiabendazole	16 ± 3	25 ± 7	23 ± 3/ (Pedras, Jha et al., 2005).
 5-Fluorocamalexin	47 ± 5	63 ± 2	76 ± 7/ (Pedras, Minic et al., 2009c).
 6-Fluorocamalexin	29 ± 10	46 ± 2	29 ± 9/ (Pedras, Minic et al., 2009c).
 5-Methoxycamalexin	51 ± 4	72 ± 1	60 ± 7/ (Pedras, Minic et al., 2009c).
 6-Methoxycamalexin	41 ± 6	63 ± 5	70 ± 4/ (Pedras, Minic et al., 2009c).

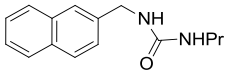
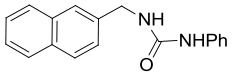
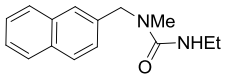
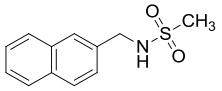
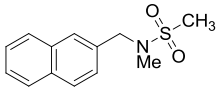
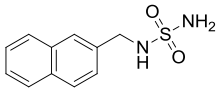
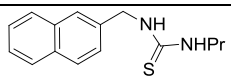
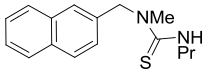
Compound	% Inhibition of BOLm		Antifungal activity at 0.10 mM/ Ref
	0.10 mM	0.30 mM	
 Brassilexin	8 ± 2	16 ± 2	8 ± 1/ (Pedras and Jha, 2005)
 5-Fluorobrassilexin	7 ± 4	14 ± 6	24 ± 19/ (Pedras and Jha, 2005)
 6-Fluorobrassilexin	22 ± 3	40 ± 5	64 ± 1/ (Pedras and Jha, 2005)
 5-Chlorobrassilexin	21 ± 6	40 ± 2	n.i./ (Pedras and Jha, 2005)
 6-Chlorobrassilexin	56 ± 5	66 ± 7	69 ± 6/ (Pedras and Jha, 2005)
 5-Bromobrassilexin	30 ± 5	45 ± 2	60 ± 10/ (Pedras and Jha, 2005)
 6-Bromobrassilexin	42 ± 4	63 ± 4	c.i./ (Pedras and Jha, 2005)
 5-Methoxybrassilexin	5 ± 1	18 ± 4	14 ± 1/ (Pedras and Jha, 2005)
 6-Methoxybrassilexin	15 ± 2	38 ± 4	n.i./ (Pedras and Jha, 2005)

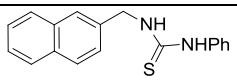
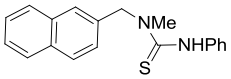
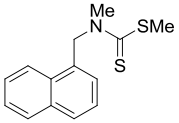
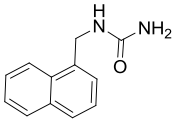
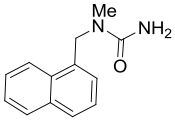
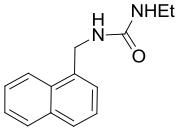
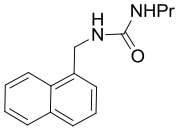
Compound	% Inhibition of BOLm		Antifungal activity at 0.10 mM/ Ref
	0.10 mM	0.30 mM	
 Sinalexin	n.d.	n.d.	24 ± 7/ (Pedras and Jha, 2005)
 Wasalexins	8 ± 5	14 ± 4	36 ± 5/ (Pedras and Suchý, 2006)
 Dihydrowasalexin	n.d.	n.d.	41 ± 8/ (Pedras and Suchý, 2006)
 *N´*-Methylbrassinin	n.d.	-	74 ± 2/ (Pedras and Jha, 2006).
 *N*-(Indol-3-ylmethyl)-*N´*-methylurea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-(Indol-3-ylmethyl)-*N*-methyl-*N´*-ethylurea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-(Indol-3-ylmethyl)-*N*-methyl-*N´*-propylurea	n.d.	-	n.d./ (Pedras and Jha, 2006)

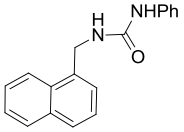
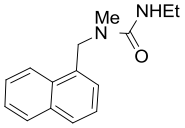
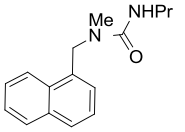
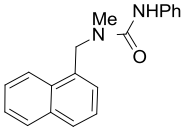
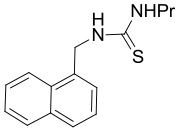
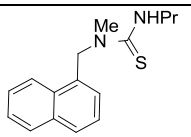
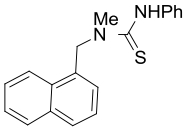
Compound	% Inhibition of BOLm		Antifungal activity at 0.10 mM/ Ref
	0.10 mM	0.30 mM	
 *N*-(Indol-3-ylmethyl)-*N*-methyl-*N´*-phenylurea	n.d.	-	30 ± 3/ (Pedras and Jha, 2006)
 Indol-3-ylmethylurea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-(Indol-3-ylmethyl)-*N´*-ethylurea	n.d.	-	21 ± 5/ (Pedras and Jha, 2006)
 *N*-(Indol-3-ylmethyl)-*N´*-propylurea	n.d.	-	43 ± 5/ (Pedras and Jha, 2006)
 *N*-(Indol-3-ylmethyl)-*N´*-phenylurea	n.d.	-	22 ± 3/ (Pedras and Jha, 2006)
 Methyl 2´-methylindol-3-ylmethylcarbamate	n.d.	-	n.d./ (Pedras and Jha, 2006)
 Indol-3-ylmethylthiourea	n.d.	-	n.d./ (Pedras and Jha, 2006)

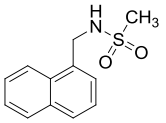
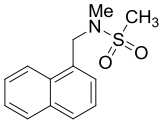
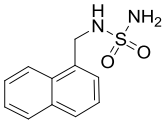
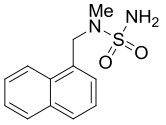
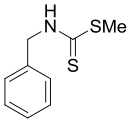
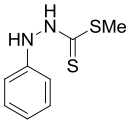
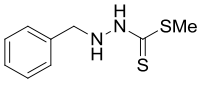
Compound	% Inhibition of BOLm		Antifungal activity at 0.10 mM/ Ref
	0.10 mM	0.30 mM	
 *N*-(Indol-3-ylmethyl)-*N´*-propylthiourea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-(Indol-3-ylmethyl)-*N´*-phenylthiourea	n.d.	-	64 ± 3/ (Pedras and Jha, 2006)
 *N*-(Indol-3-ylmethyl)-*N*-methyl-*N´*-phenylthiourea	n.d.	-	73 ± 3/ (Pedras and Jha, 2006)
 *N*-(Indol-3-ylmethyl)methanesulfonamide	n.d.	-	22 ± 3/ (Pedras and Jha, 2006)
 *N*-(Indol-3-ylmethyl)-*N*-methyl methanesulfonamide	n.d.	-	n.d./ (Pedras and Jha, 2006)
 Indol-3-ylmethylsulfamide	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-(Indol-3-ylmethyl)-*N*–	n.d.	-	n.d./ (Pedras and Jha, 2006)

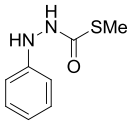
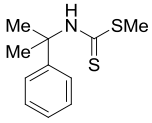
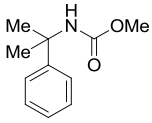
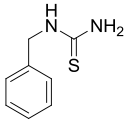
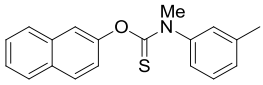
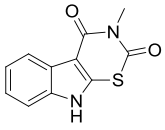
Compound	% Inhibition of BOLm		Antifungal activity at 0.10 mM/ Ref
	0.10 mM	0.30 mM	
methylsulfamide			
 Indolyl-3-propanamide	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N´*-Methyl-indolyl-3-propanamide	n.d.	-	n.d./ (Pedras and Jha, 2006)
 3-Indolylmethylpropargylamine	n.d.	-	-
 Methyl tryptaminedithiocarbamate	n.d.	-	n.d./ (Pedras and Okanga, 2000)
 Tryptophol dithiocarbonate	n.d.	-	20 ± 7/ (Pedras and Jha, 2006)
 Naphthalen-2-ylmethylurea	n.d.	-	81 ± 3/ (Pedras and Jha, 2006)
 *N*-Methyl-N-(naphthalen-2-ylmethyl)urea	n.d.	-	24 ± 2/ (Pedras and Jha, 2006)
	n.d.	-	18 ± 4/ (Pedras and Jha, 2006)

Compound	% Inhibition of BOLm		Antifungal activity at 0.10 mM/ Ref
	0.10 mM	0.30 mM	
N´-Ethyl-N-methyl-*N*-(naphthalen-2-ylmethyl)urea			
 *N*-(Naphthalen-2-ylmethyl)- *N´*-propylurea	n.d.	-	24 ± 3/ (Pedras and Jha, 2006)
 *N´*-Phenyl-*N*-(naphthalen-2-ylmethyl)urea	n.d.	-	58 ± 2/ (Pedras and Jha, 2006)
 *N´*-Ethyl-N-(naphthalen-2-ylmethyl)urea	n.d.	-	23 ± 3/ (Pedras and Jha, 2006)
 Naphthalen-2-ylmethanesulfonamide	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-Methyl-*N*-(naphthalen-2-ylmethyl)methanesulfonamide	n.d.	-	n.d./ (Pedras and Jha, 2006)
 Naphthalen-2-ylmethylsulfamide	n.d.	-	n.d. (Pedras and Jha, 2006)
 *N* -(Naphthalen-2-ylmethyl)-*N´*-propylthiourea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N* -Methyl-N-(naphthalen-2-ylmethyl)- *N´*-propylthiourea	n.d.	-	n.d./ (Pedras and Jha, 2006)

Compound	% Inhibition of BOLm		Antifungal activity at 0.10 mM/ Ref
	0.10 mM	0.30 mM	
 *N* -(Naphthalen-2-ylmethyl)-*N´*-phenylthiourea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N* -Methyl-N-(naphthalen-2-ylmethyl)- *N´*-phenylthiourea	n.d.	-	n.d. (Pedras and Jha, 2006)
 Methylnaphthalen-2-ylmethyldithiocarbamate	n.d.	-	n.d./ (Pedras and Jha, 2006)
 Naphthalen-1-ylmethylurea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-Methyl-*N*-(naphthalen-1-ylmethyl)urea	n.d.	-	28 ± 2/ (Pedras and Jha, 2006)
 *N´*-Ethyl-N-(naphthalen-1-ylmethyl)urea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N* -(Naphthalen-1-ylmethyl)-*N´*-propylurea	n.d.	-	n.d./ (Pedras and Jha, 2006)

Compound	% Inhibition of BOLm		Antifungal activity at 0.10 mM/ Ref
	0.10 mM	0.30 mM	
 *N* -(Naphthalen-1-ylmethyl)- *N´*-phenylurea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N´*-Ethyl-N-methyl- *N* -(naphthalen-1-ylmethyl)urea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-Methyl-N-(naphthalen-1-ylmethyl)- *N´*-propylurea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-Methyl-N-(naphthalen-1-ylmethyl)- *N´*-phenylurea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N* -(Naphthalen-1-ylmethyl)- *N´*-propylthiourea	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-Methyl-N-(naphthalen-1-ylmethyl)- *N´*-propylthiourea	n.d.	-	n.d./ (Pedras and Jha, 2006)
	n.d.	-	n.d./ (Pedras and Jha, 2006)

Compound	% Inhibition of BOLm		Antifungal activity at 0.10 mM/ Ref
	0.10 mM	0.30 mM	
N-Methyl-N-(naphthalen-1-ylmethyl)- *N´*-phenylthiourea			
 *N* -(Naphthalen-1-ylmethyl)methanesulfonamide	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-Methyl-*N*-(naphthalen-1-ylmethyl)methanesulfonamide	n.d.	-	n.d./ (Pedras and Jha, 2006)
 Naphthalen-1-ylmethylsulfamide	n.d.	-	n.d./ (Pedras and Jha, 2006)
 *N*-Methyl-*N*-(naphthalen-1-ylmethyl)sulfamide	n.d.	-	n.d./ (Pedras and Jha, 2006)
 Methyl *N*-benzyldithiocarbamate	n.d.	-	n.d./ (Pedras and Jha, 2006)
 Methyl-3-phenyldithiocarbazate	n.d.	-	32 ± 2/ (Pedras and Jha, 2006)
 Methyl-3-benzyldithiocarbazate	n.d.	-	-

Compound	% Inhibition of BOLm		Antifungal activity at 0.10 mM/ Ref
	0.10 mM	0.30 mM	
 Methyl-3-phenylthiocarbazate	n.d.	-	-
 Methyl*N*-(α,α´-dimethylbenzyl)dithiocarbamate	n.d.	-	-
 Methyl*N*-(α,α´-dimethylbenzyl) carbamate	n.d.	-	-
 Benzylthiourea	n.d.	-	-
 Tolnafthate	n.d.	-	-
 2-Bromothiazole	n.d.	-	-
 Rutalexin	n.d.	-	Not soluble/ (Pedras, Montaut et al., 2004b).

A1E References

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